Trends in Pharmaceutical and Food Sciences I

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PREFACE

Trends in Pharmaceutical and Food Sciences I is the first issue of an open access E-book devoted to scientific and technical research that covers the entire spectrum of drug and food research, including medicinal chemistry, pharmacology, drug delivery, microbiology and biochemical studies, as well as relevant developments in nutrition, food safety and analytical innovation.

The first chapter, Different techniques to detect G protein coupled receptor heteromers, describes state of the art techniques based in biophysical principles applied to detect oligomeric aggregates formed by G-protein coupled receptors in the cell membrane, and describes how the knowledge generated can be applied to design new compounds for the treatment of neurological and mental diseases.

Obesity has become a worldwide problem and it is well known that causes and exacerbates many health problems by promoting profound changes in physiological functions. Chapter 2, Analysis of the role of diet in the appearance of neurodegenerative processes, reviews the consequences of these metabolic alterations while considering their effects in the development of Type 2 Diabetes Mellitus, and their role in the appearance of cognitive impairments such as the sporadic forms of Alzheimer's disease.

The development of new cultivars facing climate change is an issue of great interest for the agrochemical industry and can be approached in different ways. Chapter 3, Arabidopsis Thaliana A Model for the Study of Plant Speciation, reviews different aspects of the plant immune system and the different layers of the plant immune response and signaling. The emerging field in plant research that studies how soil microbiota influences plant basic mechanisms is also discussed.

Chagas disease is endemic in Latin America, but recently and due to human migrations, it is becoming a global health problem. In chapter 4, Trypanosoma cruzi infection diagnosis: New insights, challenges and perspectives, a group of experts from several institutions describe the different techniques that can be used for the serological diagnosis of the infection and the characterization of Trypanosoma cruzi, discuss the advantages and drawbacks of each method and propose improvements that would entail important savings for health institutions.

Chapter 5, Nutrients, Control of Gene Expression and Metabolic Homeostasis, focuses on the molecular mechanisms that control metabolism by means of regulating gene expression in response to dietary inputs, to design new therapeutic strategies based on nutritional interventions against metabolic diseases. In this context, involvement of FGF21 hormone in the regulation of lipid metabolism during amino acid starvation is described, thus reinforcing its important role as an endocrine factor in coordinating energy homeostasis under a variety of nutritional conditions. This raises the possibility of dietary modulation of circulating levels of FGF21 as an alternative approach to its pharmacological administration.

Biodegradable polymeric nanoparticles encapsulating neuroprotective drugs have enormous potential to treat neurodegenerative diseases, including Alzheimer´s disease and glaucoma. Recent advances in the field are described in chapter 6, Polymeric nanoparticles for the treatment of neurodegenerative diseases: Alzheimer's disease and glaucoma, specifically the preparation of engineered polymeric nanoparticles with attached peptides or antibodies to increase their bioavailability, favoring their transport through the blood brain barrier and the blood retinal barrier, avoiding at the same time possible drug adverse and toxic effects.

The last chapter, Pentacyclic triterpenes in table olives: Determination of their composition and bioavailability by LC-M, is a review on the pentacyclic triterpenes contained in table olives, natural compounds of enormous interest due to their beneficial effects on human health, including hepatoprotective, anti-diabetic, antiviral, cardioprotective and antitumor activities. Authors describe a selective and sensitive LC–MS method for the simultaneous determination of the main triterpenic compounds present in Olea europaea L. This opens the possibility to bioavailability studies after consumption of different foods, or administration of plants widely used in traditional medicine, with the aim of studying in depth the beneficial effects of these compounds in human beings.

We hope that this new volume will attract the interest of all the scientific community, especially those working in the fields of pharmaceutical, medical, biological, chemical and food sciences.

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Contents

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Chapter 1: Different Techniques to Detect G Protein Coupled Receptor Heteromers

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Abstract

Identification of protein-protein interactions at the plasma membrane level is essential to comprehend the properties of molecular networks controlling intercellular communication. Since the nineties, it was believed that G protein coupled receptors (GPCR) acted as individual units. Resonance energy transfer techniques have been fundamental in demonstrating the existence of protein-protein interactions, discovering that GPCR can form oligomeric functional units. Sequential resonance energy transfer technique (SRET) has been designed by combining BRET and FRET to demonstrate trimeric complexes formation; and by combining Bimolecular Fluorescence complementation (BiFC) and BRET, BRET with BiFC it has been created a new technique to demonstrate tetrameric complexes. These are two invaluable techniques to identify higher order complexes, which will enable us to better understand how signals are integrated at the molecular level. The use of these techniques should help to design novel compounds for treatment of neurological and mental diseases.

Keywords: GPCR; Heteromers

Abbreviations: GPCR: G Protein Coupled Receptors; SRET: Sequential Resonance Energy Transfer; BIFC: Bimolecular Fluorescence Complementation; 7TM: Seven Transmembrane Domains; CNS: Central Nervous System; RET: Resonance Energy Transfer; FRET: Fluorescence Energy Transfer; BET: Bioluminescence Energy Transfer Technique; BiFC: Bimolecular Fluorescence Complementation; YEP: Yellow Fluorescent Protein; RFP: Red Fluorescent Protein.

Introduction

G-protein coupled receptors (GPCR) or seven transmembrane domains (7TM) are the largest and most versatile group of cell surface receptors involved in signal transduction [1]. GPCRs are encoded by a large family of genes; in the case of the human genome, they are more than 1000 proteins of which more than 90% are expressed in the Central Nervous System (CNS) [2].

GPCR are activated by a wide variety of ligands, both endogenous and exogenous, including hormones, peptides, amino acids, ions and photons of light. These receptors transduce the signal through a large number of effectors such as adenylate cyclase, phospholipases or ion channels, among others. They perform many functions in the CNS and in the periphery, controlling biological processes such as proliferation, cell survival, metabolism, secretion, differentiation, inflammatory and immune responses or neurotransmission [3,4] (Figure 1).

The G protein-coupled receptors owe their name to the interaction with the heterotrimeric G proteins, constituted by the subunits α (39-46 kDa), β (37 kDa) and γ (8 kDa). When the receptor is activated by a ligand, conformational changes

are induced that are transmitted from the receptor to the G protein that cause the α subunit to release GDP and bind a GTP. This action allows for a conformational change between the α subunit and the βγ subunits, altering their structure. Both the Gα subunit and the Gβγ complex are signaling molecules that, acting with different effector molecules, can activate or inhibit a large variety of second messengers. The signal ends when the intrinsic GTPase activity of Gα hydrolyses GTP to GDP and phosphate [5].

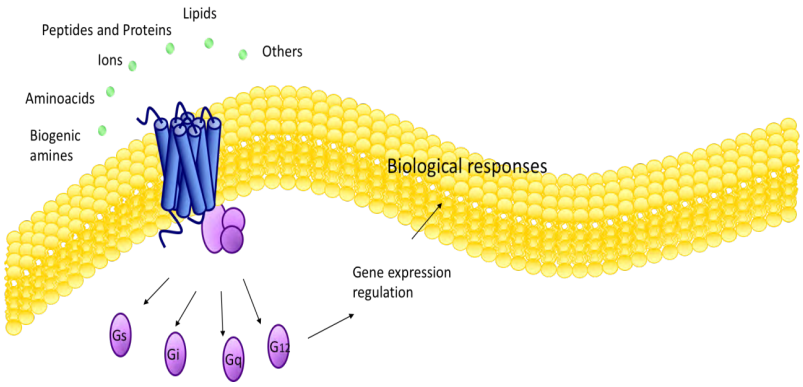

Figure 1: Endogenous ligands and cellular signaling mechanisms responsible for the various biological functions of the G protein-coupled receptors.

There are four large families for the Gα subunit in mammals, which are characterized by their primary structure and by the signaling cascade they activate [6]. The Gαs family stimulates adenylate cyclase, Gαi/o inhibits adenylate cyclase, Gαq / 11 activate phospholipase Cβ (PLCβ) and Gα12 / 13 regulate Rho proteins.

The structural characteristics and subcellular localization of the G protein-coupled receptors allow these receptors to interact with other proteins both on the intracellular and extracellular side of the plasma membrane, as well as to exhibit protein-protein interactions with other receptors or ion channels at the plasma membrane level [7].

GPCR have a topology that allows them to interact with a wide variety of proteins. These interactions determine the properties of the receptor, such as cell compartmentation or the selection of a signal and can promote their assembly into complexes that integrate a function. The proteins that interact with GPCR are mainly involved in the organization of supramolecular structures in which all types of receptors, proteins involved in signal transduction and even cytoskeletal proteins are included [7].

On the intracellular side, both the carboxy-terminal and the third intracellular loop of GPCR may present a considerable size. Thus, these regions are the most likely to interact with proteins involved in signaling or in subcellular localization of the GPCR, like cytoskeletal proteins or proteins related to receptor trafficking. These interactions may be transient or much more stable. An example of a cytosolic protein that interacts with GPCR would be calmodulin (CaM), a small peptide with the ability to bind to different cytoplasmic domains of different GPCR, including the C-terminal end of the adenosine A_{2A} receptor or the third intracellular loop of dopamine D_2 receptor, developing a calcium-dependent signal [8,9].

At the level of the plasma membrane, since the mid-1990s, several studies have demonstrated the oligomerization of many GPCR [2]. Nowadays, it is accepted that oligomerization is a common occurrence in the biology of these receptors and that they can form higher order homodimers, heterodimers and / or oligomers [10-12]. When a GPCR participates in an oligomer, its functional characteristics can change, thus the oligomerization confers new properties to the receptors, which establishes a possible mechanism to generate new functions in these receptors. This phenomenon has given rise to a new level of complexity that governs the signaling and regulation of these proteins.

Traditionally, the mechanisms of ligand binding and signal transduction for the G protein-coupled receptors were based on the assumption that they acted as monomers or independent proteins with 1: 1 stoichiometry with respect to their G protein. However, since 1990s several studies have demonstrated the oligomerization of numerous GPCR and the relevance that this implies for their regulation.

Certain indirect pharmacological evidence led researchers to think that the G-protein coupled receptors could act as dimers. The complex binding curves, both of agonists and antagonists of these receptors, were interpreted as evidence of a cooperativity that could be explained by interactions between monomers in dimeric or multimeric complexes [13,14].

Maggio, et al. using chimeras of α2-adrenergic receptors and muscarinic M_3 receptors composed of the first five transmembrane domains of one of the receptors and the last two domains of the other receptor and vice versa, carried out complementation and coimmunoprecipitation studies and suggested the formation of heterodimers [15]. When each chimera was expressed independently, no binding or signaling was observed after ligand exposure, but when both were co-transfected, binding and signaling was recovered for both adrenergic and muscarinic ligands. The dimerization of GPCR is not limited to homodimerization, that is, the physical association between identical proteins, but it also comprises the association of a receptor with another receptor or distinct protein, namely heteromerization. This association can take place between two monomers to form dimers or between multiple monomers to form oligomers. The term dimer is often used in the understanding that it is the simplest form of an oligomeric functional unit, due to the difficulty of distinguishing between dimers or oligomers with current techniques.

The interactions between GPCR are crucial to understand the varied cross-talk that is observed between neurotransmitter receptors. The oligomerization of receptors makes it possible to formulate hypotheses about the high degree of diversity and plasticity that is characteristic of a highly organized and complex structure such as the brain. A higher level of organization has been described by which the G protein-coupled receptors form structures composed not only of homo- or heterodimers, but by supramolecular complexes formed by several receptors and a variety of proteins that modify the activity of the receptor (RAMPs: Receptor Activity Modifying Proteins). These complexes interact both along the membrane (horizontal interactions), and through it (vertical interactions), and when activated by hormones or neurotransmitters they are redistributed in the membrane, resulting in clusters. The clusters would suppose a higher level of regulation of the receptors and associated enzymes and could be regulated by other receptors in these complexes and by other molecules that do not physically interact with them, but they do communicate with each other in the cluster [16].

The growing number of publications in this field has made it necessary to establish new definitions and provide nomenclature to the homomers and heteromers of GPCR [17,18].

Energy Transfer Techniques

One of the most used biochemical techniques for investigating GPCR dimerization has been the co-immunoprecipitation of receptors labeled with different epitopes. The first study that used this technique demonstrated the specific interaction between β2-adrenergic receptors [19]. Since then, similar strategies have been used to document the homodimerization of the metabotropic receptors mGlu5R [20], δ-opioids [21] and serotonin 5-HT2c [22], among others. Co-immunoprecipitation studies have also been used to demonstrate the heterodimerization of receptors of the same neurotransmitter, such as GABAB1 and GABAB2 [23] or as κ- and δ-opioid [24], and even among less-related receptors such as the CB_1 cannabinoid and dopamine D_2 receptors [25].

Although it is commonly used to study protein-protein interactions, co-immunoprecipitation of GPCR requires the solubilization of membranes by detergents, which can cause the formation of artifactual dimers by incomplete solubilization, due to the hydrophobic nature of these receptors. Despite all the controls used to rule out this possibility, the widespread acceptance of GPCR dimerization depended on a direct demonstration that these complexes exist in living cells. This was possible with the development and implementation of biophysical methods based on resonance energy transfer (RET).

Fluorescence Energy Transfer Technique (FRET)

In 1948 Theodor Förster formulated the theory of energy transfer by resonance [26] that was later applied to the study of GPCR interactions. This biophysical approach is based on the transfer of non-radiative energy (dipole-dipole) from a chromophore in an excited state (donor) to a nearby absorbing molecule (acceptor). In the case of the fluorescent resonance energy transfer (FRET), both the donor and the acceptor are fluorescent molecules, while in the bioluminescent resonance energy transfer (BRET) the donor it is bioluminescent, and the acceptor is fluorescent.

For this phenomenon to take place it is necessary that two requirements are met. The first is that the donor emission spectrum and the excitation spectrum of the acceptor overlap, so that part of the emission energy of the donor is transferred directly to the acceptor fluorophore, which emits as if it had been directly excited. The second requirement is that the donor and acceptor are very close in space (<100 Å or 10 nm). In addition, the efficiency of the transfer will decrease with the sixth power of the distance

(Figure 2).

It should be noted that most of the multiprotein complexes of a cell are between 10 and 100 Å [27,28]. Thus, energy transfer techniques offer a unique approach that allows the detection of protein dimerization in living cells, without disturbing the environment where this phenomenon occurs.

For the FRET technique, the different variants of the green fluorescent protein (GFP: Green Fluorescence Protein) obtained by mutation are used. These mutations confer different spectral properties, so that using two different mutants, with the appropriate spectral characteristics, fused to the proteins under study, allows us to determine if they are close enough to transfer energy. The most widely used pair for the FRET experiments is the one formed by the GFP^2 variant, that is excited at 400 nm and emits at 510 nm and the YFP (Yellow Fluorescence Protein) variant, that is excited at 485 nm and emits at 530 nm. In the FRET technique, as outlined in Figure 2, when a beam of light excites the GFP^2 protein, it emits fluorescence at 510 nm, and if both proteins are sufficiently close in space, an energy transfer will take place. YFP will emit fluorescence with a peak at 530 nm [29,30]. Few years ago, it was developed another variant of FRET technique based on the energy transmission between a yellow fluorescent protein (YFP) and a red fluorescent protein (RFP) that was named FRET^2.

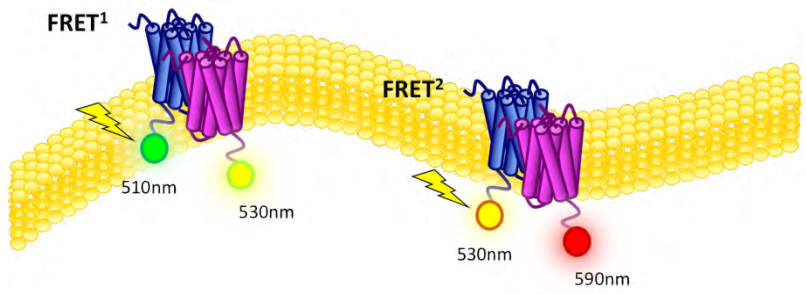

Figure 2: Schematic representation of Fluorescence Resonance Energy Transfer technique (FRET).

Bioluminescence Energy Transfer Technique (BRET)

Similar to the FRET and with the same requirements, the technique of energy transfer by bioluminescent resonance, BRET, can be considered. In this technique, bioluminescence is the result of the catalytic degradation of a certain substrate by the enzyme Renilla luciferase (Rluc) in the presence of oxygen, generating light. This light is transferred to a variant of the GFP protein, which in turn emits fluorescence at a characteristic wavelength if both proteins are close enough, indicating the dimerization of the proteins fused to Rluc and GFP [29,30].

Two variants of this technique have been described, BRET^1 and BRET^2. In BRET^1, the enzyme Rluc metabolizes the substrate coelenterazine H generating light with a peak emission of 480 nm, emission that allows excitation of the YFP protein, that will emit at 530 nm. In the BRET^2 the Deep Blue C substrate is oxidized by the Rluc, emitting light at 400 nm so that it can excite the GFP^2 protein that will emit at 510 nm (Figure 3).

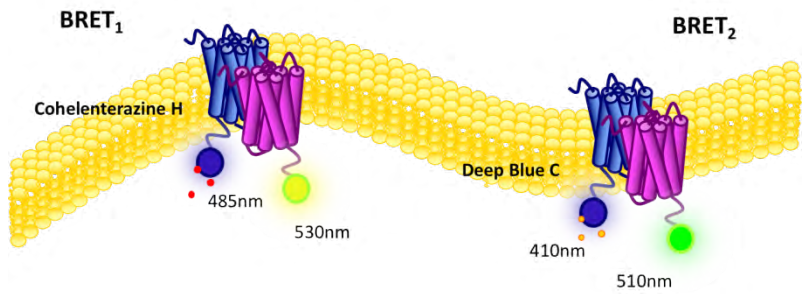

Figure 3: Schematic representation of Bioluminescence Resonance Energy Transfer technique (BRET).

The advantages of this phenomenon have been used by researchers for the study of GPCR dimerization. For this purpose, two different fusion proteins are generated and co-expressed, one of them carrying the fluorescent protein GFP or one of its variants at the carboxy terminal end of one of the receptors of interest and the other one carrying the luminescent protein Rluc at the carboxy terminal of the other receptor. These energy transfer techniques have demonstrated the existence of homodimers of the β2-adrenergic receptors [31], δ-opioid [32] and μ-opioid receptors [33], among others. A similar approach has also been carried out for the study of heteromers of G-protein coupled receptors, such as the interactions between somatostatin receptors SSTR2A and SSTR1B [34], the A_{2A} adenosine and D_2 dopamine receptors [35], the dopamine D_1 or D_2 receptors and histamine H3 [36], glutamate 2 and serotonin 2A [37] or ghrelin and oxytocin receptors [38].

Bimolecular Fluorescence Complementation (BiFC)

In recent years, variations of the FRET technique have been developed, such as FRET photobleaching or time-resolved FRET [30]. One of the most interesting results has been recently obtained using the energy microscope by resonance in milliseconds. With this technique, a conformational cross-talk between the α2-adrenergic and μ-opioid receptors has been demonstrated [39]. The binding of morphine to the μ-opioid receptor triggers a conformational change in the α2-adrenergic receptor occupied by norepinephrine that inhibits the signaling of the hormone.

The discovery of techniques such as BiFC (Bimolecular fluorescence complementation) has provided a new and very effective way to detect protein-protein interactions in living cells. This technique uses two non-fluorescent fragments of the sYFP protein (nYFP and cYFP). When the sYFP protein is reconstituted from the direct interaction between two proteins fused with these fragments, a fluorescent signal is generated [40] (Figure 4). This signal is only generated if the fusion proteins are very close in space (less than 6 nm). Later, in the same line of research, techniques have been developed that use two fragments of the Rluc protein. When the proteins fused to these fragments interact, the enzymatically active Rluc protein is reconstituted [41]. Finally, very recently, the multicolored BiFC technique (mcBiFC) has been developed that uses different fragments of different proteins facilitating the investigation of networks of regulatory protein complexes [42].

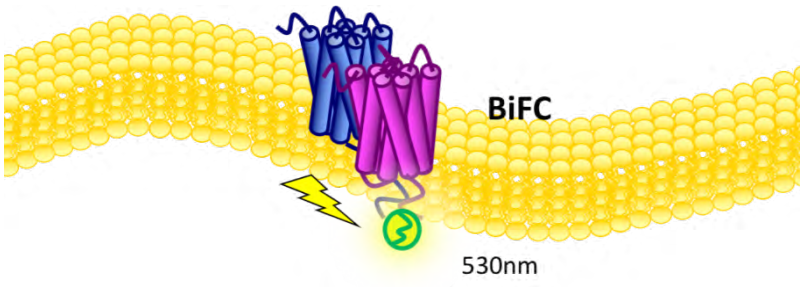

Figure 4: Schematic representation of Bimolecular Fluorescence Complementation technique (BiFC).

SRET

Since the nineties, BRET, FRET and BiFC techniques have been widely used to demonstrate the formation of a variety of GPCR heteromeric complexes in living cells. However, these techniques present some important limitations, as they can only demonstrate direct or indirect interactions between two different proteins. The demonstration of higher-order complexes involving more than two units requires the implementation of new techniques. A sequential BRET-FRET (SRET) technique allows the identification of heteromers formed by the physical interaction of three different proteins in living cells (Figure 5).

In this technique, bioluminescence is the result of the catalytic degradation of Cohelenterazine H ($BRET^1$) or Deep Blue C ($BRET^2$) substrates by the enzyme luciferase (Rluc) in the presence of oxygen, generating light. This light is transferred to the first acceptor protein, a variant of the GFP protein (YFP for $BRET^1$ or GFP^2 for $BRET^2$), which in turn emits fluorescence at a characteristic wavelength, exciting a second acceptor protein (RFP for $BRET^1$ or YFP for $BRET^2$). If all three proteins are close enough an energy transfer will be detected, indicating the existence of a heterotrimeric complex formed by the proteins fused to Rluc, GFP^2 or YFP and YFP or RFP [43].

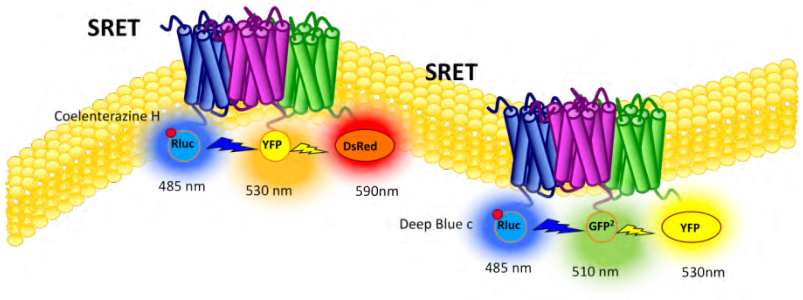

Figure 5: Schematic representation of Sequential Resonance Energy Transfer technique (SRET).

BRET with BiFC

Another technique to demonstrate heteromeric complexes of more than two receptors is the combination between BRET and Bimolecular fluorescence complementation (BiFC).

In this technique, bioluminescence is the result of the catalytic degradation of Coelenterazine H by the complementation of two non-bioluminescent fragments of the Rluc protein (nRluc and cRluc) fused to two different proteins. The reconstituted Rluc enzyme catalyzes the oxidation of coelenterazine H substrate in the presence of oxygen, generating light. This light is transferred to two non-fluorescent fragments of the sYFP protein (nYFP and cYFP) fused to different proteins. When the sYFP protein is reconstituted from the direct interaction between two interacting fused proteins, it emits fluorescence at 530 nm, demonstrating the formation of tetrameric complexes [44] (Figure 6).

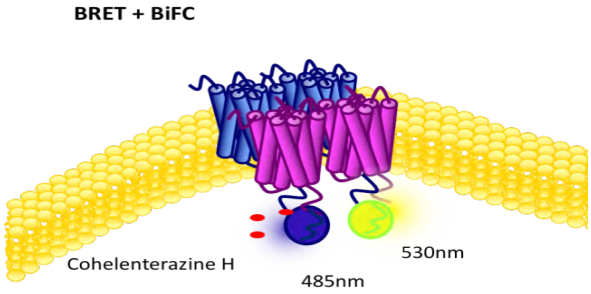

Figure 6: Schematic representation of BRET with Bimolecular Fluorescence Complementation (BRET+BiFC).

Functional Role of Dimerization

The availability of a large number of techniques for the study of GPCR dimerization has greatly facilitated the investigation of the functional role of these receptors. Dimerization is involved in the regulation of the functionality of the receptors at different levels, from the modulation of the expression of the receptors at the cell surface to the fact of conferring new pharmacological properties on the receptors forming the dimer. This has provided a new perspective to consider which is the GPCR signaling unit, as well as a new way to design drugs that act through these receptors.

Although in many cases the physiological relevance is not completely known, several studies carried out in heterologous expression systems have suggested different functional roles for GPCR oligomerization (Figure 7). For example, oligomerization may be involved in GPCR ontogenesis, that is, in the quality control of folding and membrane targeting of de novo synthesized receptors. Also, in some cases, a regulation of the formation / separation of oligomers present in the plasma membrane mediated by ligand has been observed. It has also been found that oligomerization confers pharmacological diversity, since the binding of a ligand to a receptor of the dimer can influence the binding of another ligand to the second receptor within the dimer. Oligomerization can also modify the signaling properties of a given ligand, affecting the selectivity of interaction between the corresponding receptor and its G protein, resulting in an enhancement, attenuation or coupling with another G protein. Finally, it has also been seen that oligomerization can alter the endocytic pattern for a given receptor [45] (Figure 7).

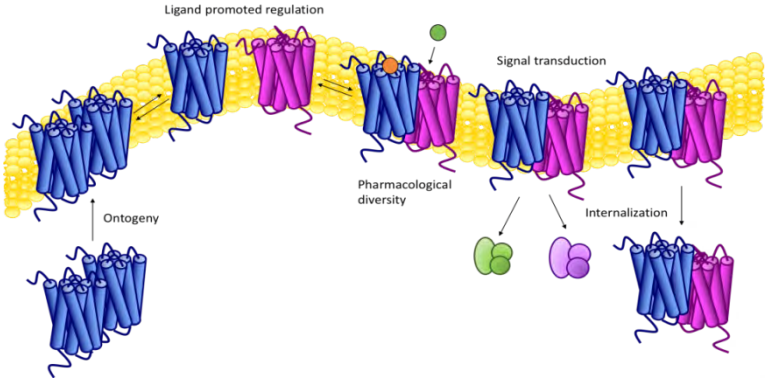

Figure 7: Oligomerization roles.

Conclusion

Resonance energy transfer techniques constitute a powerful tool to detect protein-protein interactions localized in the plasma membrane level and also in the cytosol. GPCR form molecular networks and local circuits that allow the identification of subcellular structures relevant for understanding the physiological and pathological roles of different tissues and organs. This new knowledge will provide novel therapeutic approaches for neurological diseases, mental disorders and drug addiction.

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Chapter 2: Analysis of the Role of Diet in the Appearance of Neurodegenerative Processes

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Abstract

Metabolic alterations due to the development of obesity and other diseases has become a serious problem in the world. Genetic predisposition or the intake of unbalanced diets is, in many cases, the reason for the appearance of these conditions and pathologies. In the present review, some of the consequences of metabolic alterations are explained in detail while considering their effects in the development of Type 2 Diabetes Mellitus. Also, their role in the appearance of cognitive impairments is reviewed and, the concept of Type 3 Diabetes is highly featured, as the possible mechanism for the development of neurodegenerative pathologies such as the sporadic forms of Alzheimer's disease.

Keywords: Metabolism; Diet; Obesity; Environment; Insulin; Diabetes; Cognition

Abbreviations: WHO: World Health Organization;BMI: Body Mass Index; CDCP: Centre for Disease Control and Prevention; MC4R: Melanocortin 4 Receptor; PC1: Prohormone Convertase 1; BDNF: Brain-Derived Neurotrophic Factor; T2DM: Type 2 Diabetes Mellitus; AD: Alzheimer's Disease; JNK: c-Jun N-Terminal Kinases.

Introduction

Nutrition, as it is defined by the World Health Organization (WHO), is the intake of food a person consumes considered in relationship to the dietary needs of the body. A good nutrition is defined as an adequate, well balanced diet combined with regular physical activity that allows the individual to be in good health. Poor nutrition will be the source of reduced immunity, impaired physical and mental development and reduced productivity [1]. Also, it is involved in the appearance of diseases like heart complications, stroke, cancer and diabetes [2].

Healthy diet practices begin early in life through breastfeeding. Proper nutrition on newborns will allow for better growth and cognitive development, as well as a reduction on the risk to develop overweight or obesity states during their adulthood [3,4]. In adults, the WHO has defined the standards of energy intake: total fat should not exceed

30% of the total [5] and, of that, no more than 10% should be saturated while trans-fats should not go over 1% [6,7]. Also, free sugars should not take up more than 10% [2,8].

The calculation of the body mass index (BMI) was described in order to evaluate the biometrics of an individual. The Centre for Disease Control and Prevention (CDCP; Division of Nutrition, Physical Activity, and Obesity; USA) defines BMI as the weight of a person in kilograms divided by the square of his/her height in m^2 [9]. Current standards delimit that a BMI below 18.5 kg/m^2 falls within the underweight range while values between 18.5 kg/m^2 and 25 kg/m^2 are considered normal. When the calculated number ranges between 25 kg/m^2 and 30 kg/m^2 it can be considered that the individual is overweight and, if it is 30 kg/m^2 or higher the person ought to be considered obese. Obesity is frequently subdivided in Class 1 (30-35 kg/m^2), 2 (35-40 kg/m^2) and 3 (40 kg/m^2 or higher), which is sometimes categorized as extreme or severe obesity [10].

Obesity

Over the past few years there has been a debate on how to define obesity. On the one hand, some authors believe that it is a medical condition characterized for the abnormal and excessive fat accumulation that may impair health (WHO). On the other, The World Obesity Federation argued that it should be considered a chronic relapsing, progressive disease [11]. These definitions have been the source for high controversy over the years but, in the end, it was established as a condition, since it was the best interest of patients. This statement was based on the idea that establishing it under the classification of a disease may have psychological repercussion, as well as cause for the appearance of disability labels and discriminatory behaviors in the healthcare public system [12].

Epidemiology

Nonetheless, what is clear is that obesity has become a worldwide problem. In 2017, the NCD Risk Factor Collaboration published an article after evaluating multiple epidemiological studies that had been reported between 1975 and 2016. Final data included samples from 128.9 million children, adolescents and adults and, demonstrated that in that time period there had been a three-fold increase on the prevalence of obesity. Specifically, there were over 650 million adults suffering from this condition worldwide (13% of world population) [13]. Furthermore, it was estimated that by 2016 there were 41 million children under the age of 5, and 340 million between the ages of 5 to 19, who were either overweight or obese [13].

In another epidemiological study controlled by the WHO (*MONICA* study), further evidence on obesity was gathered. According to the data, there were on average 15% of men and 22% of obese women in Europe. These percentages accounted for more than half of the adult population between 35 and 65 years [14]. Similar patterns were observed in the USA from the National Health and Nutrition Examination Surveys [15]. However, this problem is not confined on these regions. In the examinations from the NCD Risk Factor Collaboration article previously mentioned, there was an updated worldwide observation. Researchers concluded that by the year 2016 the trend on the increase of BMI has plateaued in high-income countries while it had severely accelerated in east, south and southeast Asia. Thus, if post-2000 trends keep up at the same rate it is expected that obesity numbers will continue increasing worldwide [13].

Genetics of Obesity

The contribution of genetic background to the regulation of body weight has been established over the years through the analysis of family studies, investigations on parent-offspring relationships and the study of twins and adopted children. The main conclusion that these studies report is that there is an estimate heritability of obesity of 40-70% [16]. Knowledge to date has determined that there are multiple genetic modifications that are related to the increased sensibility and genetic predisposition to develop obesity. Some of them are listed below:

- Alteration in genes encoding for the leptin receptor: Leptin is a hormone predominantly generated in the adipose tissue that regulates energy balances by controlling satiety. These modifications cause for abnormal splicing of the transcripts and thus, generate mutant forms of the receptor, lacking both transmembrane and intracellular domains. This causes for the receptor to circulate at high concentrations bound to leptin, elevating serum leptin concentrations without an actual cellular response [17]. On a clinical level, patients show normal weight at birth but exhibit rapid weight gain in the first few months of life resulting in severe obesity [18]. Deficient functionality of leptin or its receptor are associated with hypothalamic hyperthyroidism and hypogonadotropic hypogonadism [19].
- Complete deficiency for the proopiomelanocortin (*Pomc*) gene: Patients show early life hypocortisolemia leading to hypoglycaemia, prolonged jaundice, susceptibility to the effects of infection and sometimes neonatal death along with marked obesity due to hyperphagia [20, 21].
- Clinical data reports that individuals showed severe early life obesity combined with other alterations in the enteroendocrine cells due to the improper processing of gastrointestinal prohormones [23]. For example, there is the Prohormone Convertase 1 (PC1), a serine endoprotease expressed in neuroendocrine

tissues, responsible of the activation of prohormones and neuropeptides like proinsulin, proglucagon and POMC [22]. The effects of its deficiency have been reported in both, humans and mouse preclinical models. Alterations on the functionality and expression of PC1 cause high mortality rates in embryos and, in those who survive, there is a significant reduction in their size and development due to the reduced productivity of growth hormone [24].

- As another example, the deletion of the Melanocortin 4 receptor (MC4R), a receptor linked to the control of energy balance in rodents [25], also causes severe obesity in homozygous mice. Heterozygotes show body weight values intermediate between wild-type and homozygous [26]. In humans, the first report of these alterations was in 1998. It was described that mutations of *Mc4r* are highly dominant and have the same pro-obesity effects [27,28]. Since then there have been multiple descriptions of human heterozygous mutations [29-31].
- Also, there is the neurotrophin receptor TrkB. The brain-derived neurotrophic factor (BDNF) is the ligand to this receptor and has been shown to be associated with the activity of leptin in mechanisms of brain plasticity. Also, it has been demonstrated that deficiencies in the functionality of the TrkB receptor cause increases in body weight [32].

Environmental Factors

The key environment-related components that influence obesity are energy expenditure and intake. Both elements need to be levelled up in order to avoid a tendency towards body weight gain or loss. Only in the year 2000, 15% of deaths that occurred in the USA were due to excess weight, resulting from poor diet and physical inactivity [33].

Energy expenditure includes the energy spent on regular metabolic activities of the body and physical activity, which can represent 20-50% of the total expenditure. In developed countries there is a significant relationship between low levels of physical activity and obesity [34].

Energy intake must be analysed further. Food availability and portion size is one way in which the environment promotes obesity. By providing more frequent and larger opportunities in food ingestion, societies have increased their tendency towards body weight increase [35]. Also, the ingestion of high-fat and high-sugar content diets has proven to have significant pro-obesity effects [36].

Consequences

Whether it is derived from a genetic background or an environmental effect, the main problem with obesity is that it causes and exacerbates many health problems by promoting profound changes in physiological functions [37]. This relationship is approximately linear for a range of BMI indexes lower than 30 kg/m^2 but becomes greatly increased for those subjects with higher BMI values, independently of gender [37]. In particular, it has been classically associated with the development of coronary heart diseases, certain forms of cancer, sleep-breathing disorders and type 2 diabetes mellitus (T2DM) [38].

Overall, obesity causes alterations in cellular glucose metabolism in tissues like muscle, liver and others. In initial stages, the pancreas compensates for these alterations by creating a state of hyperinsulinemia, allowing for increased response by the cell. But, over time, and with increasing concentrations in free fatty acids and insulin, a state of insulin resistance appears due to the insulin receptor internalization and a reduction of its signalling by the upregulation of multiple cellular inhibitory mechanisms that eventually become the disease known as T2DM [37].

T2DM, formerly known as non-insulin dependent or adult-onset diabetes, is a chronic, metabolic disease characterized by elevated levels of blood glucose which leads over time to serious damage to the heart, blood vessels, eyes, kidneys and nerves. Symptoms are similar to those of Type 1 diabetes mellitus (unexplained weight loss, high hunger and thirst, fatigue…) but are often less marked or absent. As a result, the disease may go undiagnosed for several years until complications have already arised [39]. Epidemiological data on adults has shown that in 1980 there were 108 million diagnosed patients with T2DM and, this number had increased to 422 million by 2014 which accounted for 1 for each 11 adults worldwide [39]. Worryingly, for many years this disease was mostly reserved to adults, but it has severely increased its prevalence in children due to their upward trend to develop conditions like obesity [40,41].

Another obesity-related complication is the chronic development of low-grade proinflammatory responses due to the increased plasma levels of C-reactive protein and inflammatory cytokines, among other molecules [42]. These have been highly correlated with affectations in insulin signalling that would further aggravate the situation of insulin resistance previously described [42], as well as vascular and endothelial dysfunctions related to many other pathologies [43]. There is also evidence that the activation of macrophages in the adipose tissue is strongly associated with complications in obesity both in rodents and in humans [44]. Finally, significant evidence has been reported on the negative effects of obesity on gut microbiota [45]. These alterations are believed to be a source of further increases in body weight, proinflammatory responses and impairments

in the proper functioning of the insulin receptor [46].

In the end, these dysfunctions do not only affect peripheral tissues. Many theories have been proposed to link the appearance of metabolic dysregulations, specially, alterations to the sensitivity of insulin, with the development of neurodegenerative processes in the central nervous system [47,48]. There is evidence that supports how obesity favours cognitive impairment [49-52]. In fact, the Whitehall II study reported that obesity at 50 years of age was associated with high risk of dementia [53]. One of the main pathologies that would be the result of these alterations is the late-onset or sporadic form of Alzheimer's disease (LOAD) under the hypothesis of Type 3 Diabetes.

Type 3 Diabetes and Alzheimer's Disease

Alzheimer's disease (AD) is a relentless neurodegenerative pathology that englobes 70% of clinically diagnosed dementias [54]. Classically, it has been characterized by the presence of insoluble amyloid β (Aβ) accumulations (senile plaques) and the presence of neurofibrillary tangles derived from the hyperphosphorylation of TAU protein [54].

It has been described that Aβ soluble oligomers have high cytotoxic effects [55,56]. Yet, even though Aβ is a clearly relevant element of the neuropathology of AD, it may not be its cause but, merely, an aggravator that becomes overproduced due to aging and other alterations. Multiple data have been reported that would disprove the amyloidogenic hypothesis. For example, there are many studies of patients that showed clear clinical features of the pathology but showed none or very few Aβ depositions in the brain and, healthy aged individuals with significant numbers of senile plaques [57]. Furthermore, it was observed that 95-97% of patients had no genetic background that predisposed them to suffer the disease (genetic or early-onset disease). Actually, it seemed that in the vast majority of them the cognitive impairment of LOAD had a multifactorial origin [58]. These controversies and new observations fed the idea that there may be other theories to explain the origin of the pathology. In fact, over the years many theories have been already postulated.

The metabolic hypothesis of AD describes how alterations in the periphery affect negatively the functionality of the central nervous system. Specifically, many of these studies have been focused on the activity and sensitivity of the insulin receptor, which is central to the proper activity of neurons [59,60]. Dr. Siegfried Hoyer started presenting data on this line of research in 1985 and reported a clear link between the insulin receptor and a posterior appearance of LOAD [61]. Later on, in 1999 The Rotterdam study, an epidemiological trial conducted in the Netherlands, showed a clear causative relationship between those patients that suffered from T2DM and their risk to develop AD [62].

Moreover, modern diagnostic clinical techniques allowed for examinations and comparative evaluations of the metabolism of glucose in the brain between healthy patients and those diagnosed with AD, where a clear decline and impairment was observed [63]. Also, in a study conducted by Grillo and colleagues, they administered anti-insulin receptor lentiviral particles into the hippocampus of Sprague-Dawley rats and showed how it caused severe impairments in the cognitive capabilities of the animals after behavioural examinations through the Morris Water Maze test [64].

In the end, the idea of considering AD as a neuroendocrine disorder based on the alteration of the metabolism of glucose was labelled under the name of Type 3 Diabetes [65]. Specifically, this concept corresponds to a chronic insulin resistance plus insulin deficiency state that is mostly found in the brain but, can overlap with T2DM, representing a major pathogenic mechanism of AD neurodegeneration [66] (Figure 1).

Hence, multiple therapies that modulate metabolism have been proposed to alleviate and treat AD-affected patients. Initial studies proposed the intranasal administration of insulin but, results in clinical trials were not significantly relevant. Worse, patients developed insulin desensitization because of the overstimulation on the receptor [67,68]. Moreover, antidiabetic drugs like metformin have been proposed for the treatment of the disease and clinical results seem favourable [69]. Other research groups proposed the use of agonists of the glucagon-like peptide 1 like liraglutide and lixisenatide or, multi-target molecules that would modulate other targets like glucagon or the glucose-dependent insulinotropic polypeptide.

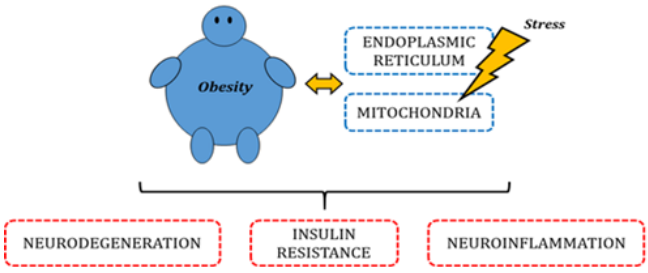

Figure 1: The paradigm of T3D establishes a relationship between the development of LOAD and alterations in the metabolism, specially linked to phenomena of insulin resistance. Obesity and the metabolic complications associated with it, increase the risk for the appearance of neurodegenerative pathologies in advanced age.

Finally, our research group has been studying the c-Jun N-terminal Kinases (JNK) as possible new targets to treat

LOAD. The JNKs are a subfamily of the Mitogen Activated Protein Kinases that respond to cellular stimuli and regulate multiple mechanisms within the cell [70]. There are three isoforms (JNK1, JNK2 and JNK3) which have differential bodily distributions and functions. In a recent publication from our group we showed how the lack of JNK2 through genetic modification caused increases in body weight, a decrease in insulin sensitivity and, eventually, cognitive affectations through different cellular mechanisms [71]. Also, already published data from JNK3 transgenic animals show that this alteration produces severe increases in body weight and metabolic affectations in high fat diet-induced obesity preclinical models [72]. Thus, our main interest focuses on JNK1. Reported data has already established that its ablation favours a reduction of body weight, as well as an increase in insulin sensitivity through the modulation of mechanisms like the activation of the insulin receptor [73,74] (Figure 2). Consequently, it is possible that inhibition of JNK1 would also provide benefits in the prevention and amelioration of neurodegenerative pathologies. Our research group has evidenced that licochalcone A, a product of the roots of liquorice that can specifically inhibit JNK1 activity, reduces neuronal death and seizures in a preclinical model of temporal lobe epilepsy [75].

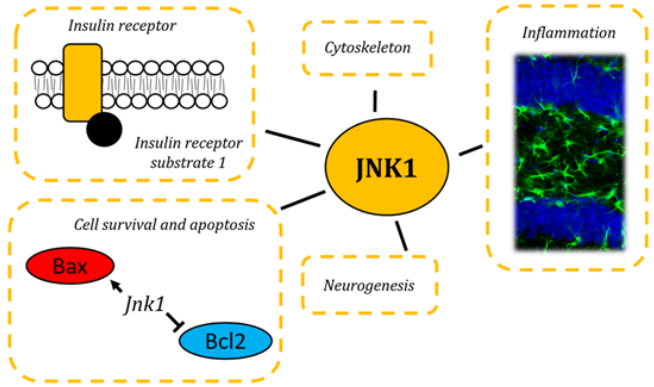

Figure 2: Some of the cellular mechanisms directly controlled by JNK1 in the brain. The activity of the insulin receptor and its substrate is highly regulated by its inhibitory phosphorylation control by JNK1. Other mechanisms may also be directly or indirectly controlled by JNK1 both in the central nervous system and in the periphery.

Conclusions

Obesity has become a major problem worldwide and new strategies need to be established in order to reduce the increasing prevalence. This condition can be the result of either a genetic predisposition or an environmental effect but, nonetheless, it is the source of many other more severe pathologies like heart failure and T2DM. Metabolic alterations have been demonstrated to affect the central nervous system and lead to the development of neuropathological dysregulations like Type 3 Diabetes and LOAD. New treatments are focusing on the modulation of metabolism in order to ameliorate cognitive impairments. Our research group proposes the modulation of JNK1 as a strategy for the future treatment of Type 3 Diabetes.

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Chapter 3: *Arabidopsis thaliana* a Model for the Study of Plant Speciation

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Abstract

The identification of the genetics and molecular bases of plant speciation is of great interest for evolutionary studies and plant breeding. The development of new cultivars facing climate change is an issue of great interest for the agrochemical industry, which finds its roots in pharmaceutical sciences. Although changes in ploidy level represent the most frequent cases of plant speciation, other more moderate changes in the genome have an identical outcome. These are the bases for a number of postzygotic reproductive isolation barriers based on the Dobzhansky-Dobzhansky-Muller (DM) model. In the recent decades it has become possible to identify genes and pathways underlying deleterious DM incompatibilities in different plant species, and in particular in Arabidopsis. Here we discuss the usefulness of this model species for investigating the bases of plant speciation and how DM incompatibilities can be attenuated by molecular approaches or even shaped by the environment.

Keywords: *Arabidopsis thaliana*; Immunity; Natural variation

Abbreviations: CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; CC: Coiled-Coil domain; DAMP: Damage-Associated Molecular Pattern; ETI: Effector-Triggered Immunity; GWAS: Genome-Wide Association Study; Hpa: Hyaloperonospora arabidopsidis; HI: Hybrid Incompatibility; LRR: Leucine-Rich Repeat; LD: Linkage Disequilibrium; NBD: Nucleotide Binding Domain (NBD); PAMP: Pathogen Associated Molecular Patterns; PTI: PAMP-Triggered Immunity; PRR: Pattern Recognition Receptors; Pst: Pseudomonas syringae; QTL: Quantitative Trait Locus; SNP: Single Nucleotide Polymorphism; TIR: Toll/Interleukin-1 Receptor domain; T3SS: Type Three Secretion System.

Introduction

The model plant model *Arabidopsis thaliana* (L.) was first discovered by Johannes Thal (1542-1583; Germany) in the sixteenth century at the Harz Mountains (Germany), and hence its specific epithet of "*thaliana*". However, the first one to summarize the potential of the species as a model for plant genetics was Prof. Friedrich Laibach [1,2], with prominent contributions from other plant researchers afterwards. *Arabidopsis thaliana* (L.) was the first plant with the whole genome sequenced and a T-DNA mutant collection developed in the beginning of this century [3].

Evolutionarily, *A. thaliana* was also the first one to diverge from its relatives Arabidopsis around 3.8-5 millions of years ago (Mya), whereas the remaining species began to separate 2 Mya [4]. For example, when *A. thaliana* is compared with its closest relative, *Arabidopsis lyrata* (L.), 56% of the genome cannot be aligned and their genome size varies by 40%. This is because *A. thaliana* has less transposable mobile elements (TE) than *A. lyrata*. These TE are important for gene expression regulation of neighboring genes.

Morphologically, *A. thaliana* has a well-defined basal rosette, with small-ovate juvenile leaves of smooth margins, and bigger ovate adult leaves with partially serrated margins. Leaves have long and simple-forked trichomes. The central inflorescence has bifurcations emerging secondary inflorescences, carrying lanceolate caulinar leaves, and white-color flowers with four sepals, four petals, six stamens and two carpels. Fruits in siliques contain about 20 seeds per fruit [5]. *Arabidopsis thaliana* has a short lifecycle and produces a large number of seeds. It has a small size but a large plasticity to adapt to different environments and growth conditions. *A. thaliana* is currently one of the best models to investigate basic biological processes: primary metabolism, defense, seed-dormancy, abiotic stress adaptation, and evolutionary biology [6–9].

Moreover, one of the main characteristics that distinguishes *A. thaliana* from other Arabidopsis relative species is the capacity of self-fertilization, being *A. thaliana* an autogamous plant, not like other Arabidopsis genus species. The capacity of self-pollination provides some short-term advantages [10]. Autogamous plants can produce more seeds than the outcrossing ones. This enables proper seed dissemination and the capacity to colonize new unoccupied niches [11]. Acquired mutations or genomic variants are maintained in the progeny and will become fixed in the population, also depending on fitness effects of such mutations. However, self-pollination produces inbreeding negative effects such as the inbreeding depression (ID) associated with the increased homozygosity [12,13].

Arabidopsis thaliana recently became a self-compatible plant through independent and gradual fixation of disabling mutations at the S-locus. S-locus codifies for two proteins: S-locus receptor kinase (SRK) on stigma epidermal surface and S-locus soluble ligand cysteine-rich protein (SCR) on pollen wall [14,15]. Loss-of function mutations at the SI-locus can trigger negative effects. One of these negative effects is the decrease in genetic recombination between individuals that can produce lower diversity and plant adaptation capacity. On the other hand, self-compatibility provides in some cases a reproductive-improvement when the pollinator is absent or lacks the synchrony of flowering-time. Hence, self-compatibility and the acquisition of loss-of-function mutations at the SI locus can be considered important for speciation by reproductive isolation [16,17]. An example for the importance of self-incompatibility breakdown for speciation are the diploid species *Capsella rubella* and *C. grandiflora* (2n = 2x = 16). Recent studies have suggested the occurrence of a self-incompatibility breakdown event in a local Greek population of *C. grandiflora*. This population was likely exposed to an extreme bottleneck and forced to self-reproduce from a single or few individuals, and to generate a fertile offspring with the consequent appearance of *C. rubella*. Finally, *C. rubella* has spread in the Mediterranean region together with agriculture practices [10].

Hereafter we summarize some aspects on the plant immune system as well the different layers of the plant immune response and signaling using as a model plant model *A. thaliana*. We focus on the hybrid incompatibilities, which can represent an incipient way of speciation by reproductive isolation. We describe some of the techniques that we currently use to suppress plant hybrid incompatibilities and to study the complex mechanism of immune signaling, epistasic interactions and the contribution in the resistance and growth tradeoff.

Natural Variation in *Arabidopsis thaliana*

Ecologic and geographic isolation drive genetic divergence and the accumulation of polymorphism. Natural variation refers to the differences in the genetic material between individuals and populations that may (or not) have adapted to deal with different local environmental conditions [18]. Humans are the unique animal species on Earth able to see and exploit natural variation for its benefit. Crosses between species have resulted in plant domestication and the advance of agriculture [19].

Arabidopsis thaliana has a worldwide geographical distribution with a strong anthropogenic influence of dispersal and colonization of new habitats [20]. It is native from the Northern Hemisphere (Eurasia) but has spread by human activity and naturalized in other geographical regions such as North and South America, Africa, Oceania, and some islands such as Canary Islands and Cape Verde [21]. *Arabidopsis thaliana* is thought to have adapted to different habitats, accumulating and maintaining beneficial traits for its ecological niche conditions. Researchers and botanists have collected individuals from natural populations (accessions). For example, Prof. F. Laibach, collected in 1920 (Spain) the Bla-5 and Sf-2 accessions in Blanes and Sant Feliu de Guixols, respectively.

During the last decades, several studies based on natural variation have been performed, resulting in a large number of genes identified that have essential biological activities. Importantly, many of these genes are not exclusive for Arabidopsis. As such, plant-breeders can extrapolate basic knowledge to cultivated plants, aiming at improving crop yields [22]. This is feasible because many pathways in development and defense have conserved mechanisms, signaling hubs or even similar genes. In conclusion, A. thaliana natural variation is considered a powerful tool to investigate the genetics and molecular changes involved in plant adaptation and genome evolution [20].

Today, a big effort is being performed to sequence a large number of accessions for evolutionary studies (https://1001genomes.org/). Next generation sequencing (NGS) techniques are providing thousands of genetic variations and markers across genomic regions, not only in Arabidopsis but also in maize, rice, soybean and wheat, among other cultivars. These data enable to apply quantitative genetics to determine casual regions underlying a measurable phenotypic trait [23].

One of the approaches to localize or map a locus associated with an observed phenotype is the quantitative trait loci (QTL) mapping. The availability of genetic markers enables to apply QTL analysis using recombinant inbred lines (RILs) or other genotyped populations suitable for such analyses. The power of QTL analysis and fine-scale mapping relies not only on the low economical cost and quickness of genetic mapping, but also on the historical recombination event produced in nature that reduces the degree of linkage disequilibrium (LD) and the tight physical distance between causal loci and the polymorphism [24]. On the other hand, QTL analysis fails when there is allelic segregation between the parental lines or when a huge number of recombination occurs in the RIL population.

LD is a nonrandom association of alleles at two or more loci in the general population and its use is crucial for genetic association studies. The chromosomic extent of LD in haplotypes is crucial because it determines how dense can be the map of the associated genomic loci [25]. LD is broken by recombination events and is shaped by local adaptation, genetic divergence, natural selection and population history. In autogamous plants with high homozygosity, such as *A. thaliana*, LD is strong because of the self-style of reproduction, whereas some events of recombination can also be found and interfere with the assumed association [26].

The strong LD in Arabidopsis produced through several self-pollination events, its genomic data availability, and the development of statistical methods, makes Arabidopsis a suitable plant for genome-wide association studies (GWAS). GWAS is a technique that uses global high-density single nucleotide polymorphism (SNP) data in a large set of accessions, and it is an alternative, or probably better to say a complementary method, to QTL mapping. Hence, GWAS is a genetic study that attempts to identify commonly occurring genetic variants (SNP) that contribute to the observed phenotype [27]. To apply the study, it is required numerical discrete or continue data because they improve the power of SNP detection. When data is a phenotypic trait with a non-numerical value, the observed trait must be categorized in numerical by categorizing it in binary (e.g. 0 = non-affected; 1 = affected) or in multiple-numerical categories. There are online-free applications that enable GWAS analyses, whereas the most powerful analyses are performed in Linux and R scripts. Nevertheless, findings obtained by GWAS must be validated by other complementary genetic approaches.

GWAS analysis has some limitations that can lead to misleading associations [28]. These involve the existence of strong and complex population structure due to isolation, accessions relatedness, genetic heterogeneity, and trait heritability and population size. Narrow sense heritability is telling us how strong contributes the genetic variant to the phenotype variation or in other words, how much connected is the genotype to the phenotype. There are many ways to measure LD, all related to the difference between the frequency of co-occurrence for two alleles and the frequency expected when the two markers are independent. Furthermore, there are two commonly used LD measures: D' and R^2 [29,30]. The statistical mixed-linear-model (MLM) method takes the population structure into account by using a genetic estimated marker data matrix and can overcome the population-structure limitation as well to predict the heritability [31,32]. Increasing the population size will improve meaningful association but, can reduce variant recovery by weakening the polymorphism-loci correlation or decreasing allelic frequency [27]. Once the analysis is performed, it is time to analyze the significance of the outcome. Hence, more statistical analyzes are applied, for example, the 5 % Bonferroni threshold [33], Q-Q plots and Manhattan plot for P value inflation [34], and false discovery ratio (FDR) [35].

In Arabidopsis, GWA studies were reported in 2010 identifying disease resistance genes against the plant pathogenic bacteria, Pseudomonas syringae [36]. Recently, GWAS has demonstrated the capacity to identify the genetic bases for defense traits not only in Arabidopsis, but also in other species such as rice, tobacco, maize, soybean and wheat, among others, and it is widely used for the identification of genes that influence other complex traits [37-41] including tolerance to high magnesium supply [42], identification of genes shaping the leaf microbiota [43], identification of new components involved in hydrogen peroxide signaling an tolerance [44], abscisic acid accumulation during abiotic stress [45], herbicide resistance in wheat [46], salt tolerance, secondary metabolites variation, morphological and yield-trait identification in rice [47,48], among others.

The Plant Immune System

Biotic stress causes between 30% to 40% of crop losses with huge economical costs, while abiotic stress has an impact of 6% to 20% [49]. In addition, biotic stress has a strong negative impact on crop yield, not only on the quality of the product but also decreasing the potential of plant growth due to the metabolic cost of maintaining an activated

immune system [50].

Plants are sessile organisms with no cellular, humoral or adaptative immune system. Their capacity to resist to pathogen colonization is based on the innate immune response and the capacity to recognize pathogens through resistance (R) proteins encoded by *R* genes or other receptors at the plasma membrane [51].

To deal with pathogen attack, the plant has different layers of defense according to the nature of the pathogen that is threating the plant. However, plant pathogens have different strategies to survive and adapt to their hosts. Pathogens can be herbivores insects, nematodes, fungi and viruses or microorganisms such as bacteria and oomycetes. Hence, fungi and bacteria have similar mechanisms to avoid plant recognition. The interference mechanism is based on the capacity to deliver into the host cell proteins known as effectors, virulence factors, in such a way that pathogen fitness is enhanced. Furthermore, the authors suggested an easy to interpret model (the zig-zag model) which summarize the plant-pathogen coevolution and the plant-immune response [52]. Actually, the model is more complex and somehow not fully appropriate for the different known mechanisms of infection and pathogenicity (hemibiotrophic and necroptrophic). Nevertheless, it is still useful to acquire a global view of the plant-pathogen coevolution (Figure 1). According to the "zig-zag model", molecules at the surface of bacteria, fungi or oomycetes, might represent molecular fingerprints called pathogen associated molecular patterns (PAMPs) and microbial associated molecular patterns (MAMPs) [53]. Recognition PAMPs or MAMPs by Pattern Recognition Receptors (PRR) results in a PTI response that arrests pathogen invasion. At a second stage, pathogen successfully use effector or avirulence proteins (Avr) that contribute to pathogen virulence by interfering in a spatial and temporal manner with PTI signaling and delay plant immune responses [54] thus resulting in effector triggered susceptibility (ETS). To counteract this, plants have developed a second line of defense comprising intracellular receptors or R proteins which mainly are nucleotide-binding site leucine-rich repeat (NB-LRR) type. Their conformational activation leads to ETI (effector triggered immunity). ETI triggers global transcriptional reprogramming, generation of ROS and RNS, induction of programmed cell death (PCD) and hypersensitive response (HR) at the infection sites [55,56].

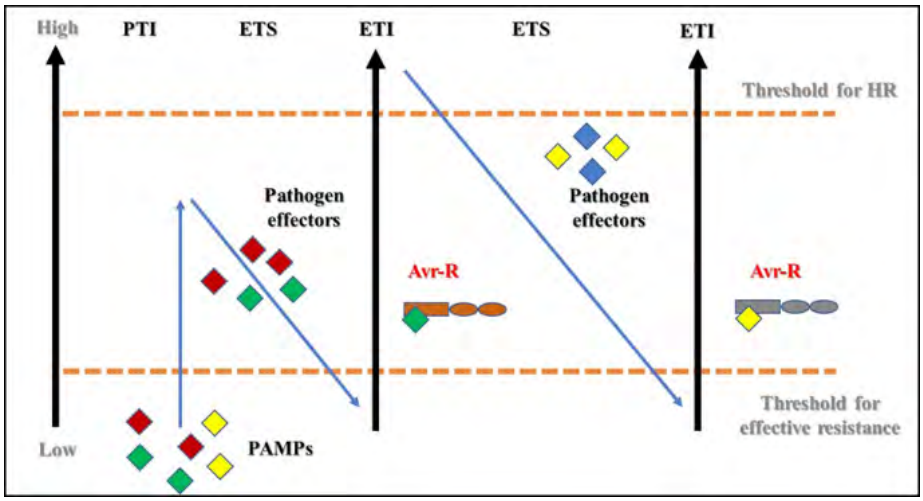

Figure 1: Zig-zag model for the illustration of the quantitative output of the plant immune-system interaction with pathogen effectors. Fingerprint molecules such as PAMPS initiate the pathogen triggered immunity (PTI) that is an intermediate response enough to block or delay pathogen invasion. At second stage, some pathogens deliver proteins, known as effectors, that interfere with the PTI response and produce the effector triggered immunity (ETI) which is a strong and broad sense immune response highlighted with the induction of plant cell death (PCD), also known as hypersensitive response (HR). Effectors can be recognized by NLR receptors initiating the effector triggered immunity (ETI). However, variations on these effectors that lead to unrecognition or the lack of specific intracellular receptor, produce the effector triggered susceptibility (ETS). Finally, due to the effector-plant receptor coevolution, the effector variant can be recognized one more time and initiate the ETI. Adapted from Jones & Dangl [52].

PAMP and DAMP Triggered Immunity

When PAMPs or MAMPs are recognized by PRRs, the PAMP-Triggered Immunity (PTI) initiates. PTI is an early and broad-range specific immune response against non-host specialized pathogens [57]. For example, bacterial PAMPs such as Flagellin 22 (flg22) or the Elongation Factor Tu 18 (elf18) are recognized by membrane anchored PRR

(FLS2 and EFR, respectively). Recognition stimulates the recruitment of the leucine-rich repeat receptor-like kinase BAK1 (brassinosteroid insensitive associated receptor kinase 1) and the receptor-like cytoplasmatic kinase BIK1 that, upon trans-phosphorylation, activates a plasma membrane-anchored specific NADPH oxidase (RBOH) and mitogen-activated protein kinases (MAPK). In A. thaliana, there are 10 different isoforms for RBOH NADPH oxidases (RbohA - J). Only isoforms RbohD and RbohF have been reported to participate during PTI and are known to trigger the oxidative burst after PAMPs/MAMPs and DAMPs recognition. RBOH activity is regulated at post-translational level by phosphatidic acid (PA), Ca^{2+}, FADH, NADPH and phosphorylation by BIK1 and CPKs (Ca^{2+}-dependent protein kinases). RBOH are responsible for ROS production or oxidative burst, whereas MAPK cascades promote the expression of R genes, biosynthesis of antimicrobial compounds and strengthening of cell wall structures [58–60].

Due to the pathogen invasive activity, cell structures are damaged. This process produces and delivers damage-associated molecular patterns (DAMPs) initiating the DAMP-triggered immunity (DTI) which enhances and maintains both PTI and ETI responses. Thus, fingerprinting molecules have different nature and involve proteins, lipids, sugars, nucleotides, cell-wall degradation-derived products and maybe also polycationic molecules such as polyamines [61-64].

Effector Triggered Immunity

Host-specialized and non-specialized pathogens have different invasive mechanisms. The non-specialized ones can only be recognized by PRR receptors and trigger PTI whereas host-specialized pathogens deliver avirulence proteins (effectors) that are recognized by intracellular nucleotide binding leucine-rich repeats (NLR) receptors that trigger ETI and hypersensitive response (HR). Very often, these intracellular receptors are not binding directly to the effectors, but they guard a host protein that is the target of such effectors. NLRs are present in plants, animals, and fungi, revealing their evolutive importance to recognize intracellular pathogens or their effectors. Plant NLR proteins can be found in angiosperms, gymnosperms, bryophytes, and liverworts (*Hepaticophyta*) but not in the unicellular alga *Chlamydomonas*. In animals, NLR receptors are found in mammals (also known as NOD-like receptors), chordates and sponges but not in nematodes or arthropods [65–68].

In gram-negative bacteria, such as *Pseudomonas syringae*, effector delivery is mediated through the Type Three Secretion System (TTSS) [54]. An example of the guard-guardee model is RIN4, a plasma-membrane associated protein that is target of TTSS effectors AvrRpm1, AvrB and AvrRpt2. RIN4 is guarded by the NB-LRR proteins RPM1 and RPS2, which sense perturbations in RIN4 homeostasis. When AvrRpm1 or AvrB target RIN4, this protein becomes phosphorylated and activates RPM1. In contrast, AvrRpt2 cleaves RIN4 and this leads to RPS2 activation. Activation of ETI via NB-LRR receptors induces an HR response at the site of infection and promotes disease resistance [52,69-71]. Filamentous pathogens can deliver effectors from the intercellular space by hyphae or haustoria [72]. *Hyaloperonospora arabidopsidis* (*Hpa*) is an obligate biotrophic oomycete that grows under high humidity and can infect *A. thaliana* [73,74].

Arabidopsis has NB-LRR receptors (Recognition of *Peronospora parasitica* 1, *RPP1*) that recognize allelic variants of the *Hpa* ATR1 effector. Some additional receptors show sequence similarity to *RPP1* and are referred to as *RPP1-like* [75,76], although their recognition specificities are still unknown [77]. Furthermore, it is widely accepted that NB-LRR mediated immune responses are only effective in obligate biotrophs (e.g. *Hpa*, *Ustilago spp*., *Uromyces*., *Phytophthora spp*.) that require living hosts to exploit their metabolites, but not effective against necroptrophic pathogens (e.g. *Botrytis spp*., *Fusarium spp*., *Sclerotinia spp*., *Pythium spp*.). Necrotrophs kill and destroy the host by exhaustive use of the organic material and nutrients for its own growth [78]. Moreover, some hemibiotrophic pathogens establish a biotrophic interaction with the host at the beginning but switch to a necrotrophic lifestyle afterwards [79].

NLR Regulation and Signaling

NLR receptors can be categorized according to their N-terminal domain. TIR-NB-LRR (TNL) receptors carry a toll-interleukin-1 domain in their N-terminus. CC-NB-LRR (CNL) carries a coiled-coil domain in their N-terminus and RPW8-NB-LRR (RNL) has a powdery mildew-8 domain [80]. TIR and CC domains of NLR receptors are required for downstream signaling and can trigger cell death [81,82]. The NB domain contains STAND motifs with a regulatory activity via ATP hydrolysis that induces protein conformation changes [83]. Between the NB domain and the LRR, an interspace separating sequence can be found (NL-LINKER). Structural variation of the NL-LINKER sometimes correlates with NLR activity, suggesting an intrinsic variation that modulates receptor activity [84]. The C-terminal sequence of NLR is composed of several leucine-rich repeats (LRR) domains. This region is highly variable and can bind effector proteins and interact with the NB domain thus inducing the conformation changes required for activation [82,84]. Mutations in the different NLR domains can affect receptor activity and lead to self-activation, incapacity to bind molecules (e.g. effectors and nucleotides) or to oligomerize and trigger an autoimmune response.

TNL signaling requires the nucleocytoplasmatic lipase-like protein, enhanced disease susceptibility 1 gene (*EDS1*), its coreceptor phytoalexin deficient 4 (*PAD4*) and senescence associated gene 101 (*SAG101*) that promotes SA biosynthesis by stimulating salicylic acid induction deficient 2 (*SID2*) / isochorismate synthase 1 (*ICS1*) gene expression [85,86]. SA and EDS1/PAD4 have a positive feed-back relationship. CNL receptors are almost *EDS1*/*PAD4* independent, but *NDR1* dependent [87-89].

ETI activation induces a global immune response that can be transmitted throughout the plant to prevent disease [90]. This broad-spectrum immune response is the systemic acquired resistance (SAR) and requires SA gradient accumulation and conformational changes (S-nitrosylation) of the master SAR protein NPR1 (non-expressor of *PR-1*) that translocates into nuclei and promotes gene expression (via TGA transcription factors) of WRKY transcription factors, redox regulation, DNA repairment and membrane traffic readjustments [59,91-95].

The Batseon-Dobzhansky-Muller (BDM) Model of Reproductive Isolation and Plant Autoimmunity

Arabidopsis natural variation can be a useful tool to study underlying speciation processes derived from genetic divergence [96]. How natural populations with genomic variants evolve to new species without being incompatible in the ancestral population and without passing through any deep adaptive valley was an enigma for Darwin an others evolutionary biologist [97,98]. Studies have described ways of plant speciation through the occurrence of reproductive barriers at pre-zygotic or post-zygotic stages. Zygote formation can be affected during the step of pre pollination or postpollination. Handicaps of prepollination underlie ecological isolation (when fitness is reduced in heterospecific habitat), phenological isolation (e.g. differences in flowering time) and/or pollinator specialization. On the other hand, reproductive barriers after pollination are related to pollen-stigma interactions in which foreign pollen is recognized and ovule fertilization blocked [99]. Furthermore, postzygotic reproductive barriers occur when the zygote is formed but the hybrid exhibits sterility or is not viable. Hybrid seeds can also be defective, and germination or fitness strongly affected in F1 or F2 generations [98].

The BDM or Bateson-Dobzhansky-Muller [100-102] model of genetic incompatibilities explains the process of speciation through postzygotic reproductive isolation (Figure 2). The theory underlying this speciation model considers an ancestral population where two alleles (AABB) coexist with no impact on growth and fitness. When the ancestral population is split in two, alleles A and B mutate and generate new alleles (a and b) in the respective populations. Through transition states (AaBB or AABb) variants become fixed in the two populations (aaBB vs AAbb) with no costs on fitness. The problem emerges when individuals from these two diverged populations mate and evolved alleles are forced to co-exist in the same genome, leading to a lethal epistatic interaction between them that is the basis for hybrid incompatibility. Incompatible hybrids are sterile or lethal. Other symptoms such as hybrid weakness might be related to intermediate stages towards the complete reproductive isolation.

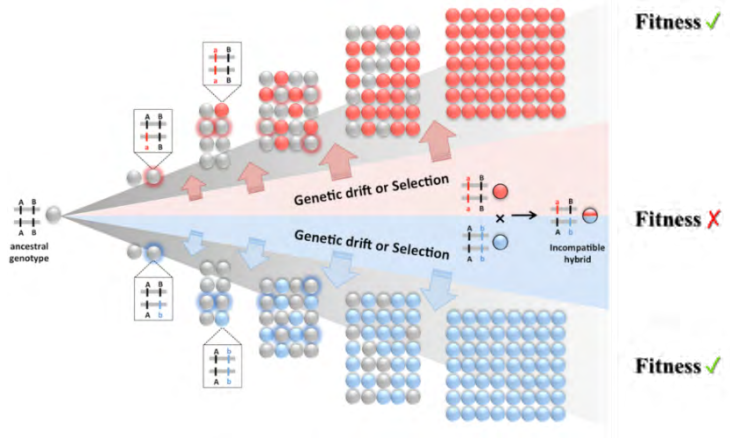

Figure 2: The Bateson-Dobzhansky-Muller (BDM) model for genetic incompatibilities. An ancestral population has two coexisting alleles (AABB). After a split, two new populations appear and exhibit independent allele divergence originating alternative alleles "a" or "b", which become fixed. When individuals from these two diverged populations are crossed, an epistasic deleterious interaction between the two new alleles occurs that leads to hybrid incompatibility. Adapted from Alcázar, et al. 2012.

There are many examples of HI in different plant species involving epistatic interactions with at least one immune *R* gene. These hybrids exhibit an autoimmune response which has a strong impact on plant growth and fitness. Dwarfism, cell death and sterility are hallmarks for immune-related. These hybrids are not only useful to study underlying mechanisms of reproductive isolation, but they also highlight molecular mechanisms that enable fine-tuning defense responses or guard-guardee relationships. Other mechanisms of hybrid incompatibility have been reported such as cytonuclear incompatibilities and differences in ploidy level [103], but these are not the focus of this study. In the recent years, immune-related HI has been focused on the identification of causal genes. Here, we have studied what are the effects from the genetic suppression of immune-related hybrid incompatibilities on plant growth, overall fitness and resistance against pathogens.

Most of the described HI in plants includes incompatible interactions between *R* genes and other immune-signaling pathway related proteins (e.g. NLR receptors, RLK, receptors like proteins, and other types of immune-signaling proteins). Among the NLR-based HI, TIR-NB-LRR receptors are the most frequent but involved although CC-NB-LRR incompatibilities have been described [104]. In rice, deleterious interactions leading to self-immune activation were described between NLR-RLK, two CNL receptors, and two RLK proteins [105,106]. In other species such as, wheat, lettuce and *Capsella* spp., incompatibilities involving CNL, RIN4, and NPR1 were reported, too [107–109]. Moreover, in Arabidopsis TNL-based incompatible interaction was described by Bomblies, et al. [96]. Alcázar, et al. [75,110–112], and Chae, et al. [113] identified additional genetic deleterious interactions often involving a common locus, RPP1-like HI (Figure 3).

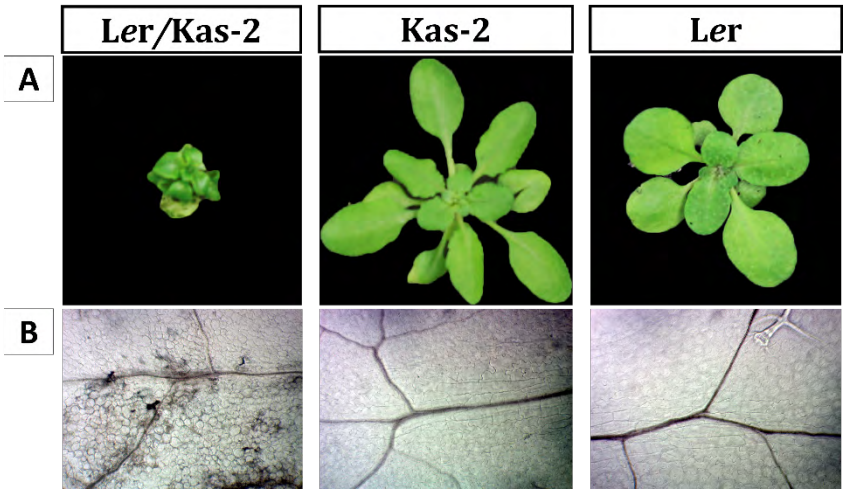

Figure 3: Temperature dependent hybrid incompatibilities between two natural A. thaliana accessions, L*er* and Kas-2. The incompatibility implies the deleterious epistasic interaction between the RLK protein, SRF3, from accession Kas-2 and the L*er* *RPP1-like* haplotype which codifies for TIR-NB-LRR receptors. When F2 hybrids L*er*/Kas-2 are grown under the Arabidopsis environmental natural temperature of 14 ºC – 16 ºC, a self-immune activation is revealed by the stunted growth or dwarfism (A), sterility and massive cell death as hypersensitive response detected by Trypan blue stain (B).

During last decades, plant breeders have used different strategies in order to obtain new plant varieties with specific commercial or adventurous traits. Plant researchers use mutagenesis techniques as basic tool to study gene function and investigate main plant physiology (Figure 4). First mutagenesis techniques were based on the use of energetic radiations such as X-ray, γ-ray and fast neutron bombardment. X-ray produces high destructive changes and, in many cases, leads to a non-viable plant. γ-ray is less destructive and causes point mutations and small deletions, whereas fast neutron bombardment is used to induce large deletions and chromosomic translocations. UV-B and UV-C are non-atomic radiations that are also used for plant mutagenesis with different degrees of success [113].

Chemical mutagenesis is an alternative. The advantage of this technique relies on the easiness of application in a basic equipped laboratory and the known nature of induced mutations. For example, the ethyl-methane sulfonate (EMS) alkylates guanine bases causing the shift GC to AT during DNA-polymerase replication. Sodium azide (Az) and methylnitrosourea (MNU) are chemicals also used in combination (Az-MNU). They induce a GC to AT or AT to GC shifts but are less stable, more toxic and tedious for work [114].

Figure 4: Ethyl-methane sulfonate (EMS) mutagenesis screen on a hybrid incompatible (HI) L*er*/Kas-2 population. Plants with a suppressed HI when are grown under long day conditions, 14 ºC – 16 ºC, 70% of relative humidity (RH), and 160 µm photons $m^{-2} \cdot s^{-1}$ of light intensity. Casual point-mutation identification can be carried out by next generation sequencing (NGS) techniques or by genetic mapping.

Finally, since the development of CRISPR/Cas9 genome editing tools in plants, specific change can be induced in a target gene with high precision (Figure 5). For instance, loss-of function mutants can be isolated when gene-mutant is not available or for non-model plants [116].

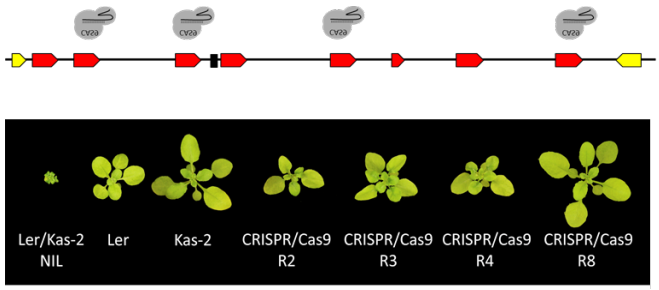

Figure 5: CRISPR/Cas-9 gene directed mutagenesis on the incompatible near isogenic line (NIL) L*er*/Kas-2. Loss of function mutations at *R2*, *R3* and *R8* suppress the occurrence of hybrid incompatibility and allows the study of individual gene contribution of the eight genes described into the L*er* *RPP1-like* haplotype as well, the motifs related with the protein functionality [115].

Plant Growth Promoting Bacteria

Recently, there is a growing interest to investigate plants in their habitats, and more specifically the study of the rhizosphere, phyllosphere and the interplay microbiota-plant growth and health. Soil microbiota plays a key role in plant immunity, pathogen resistance, water availability, nutrient assimilation, turnover and competition. It was suggested that the small microbiota community interacting with plants, in comparison with the huge quantity of taxa found in soil, can be explained by the host genotype and has an effect on plant health [117-119]. Lebeis, et al. [120] suggested that SA plays a role on the root-microbiome community composition. Castrillo, et al. [121] have demonstrated that root microbiota drives phosphate integration and immune response during phosphate starvation by regulating the master transcriptional phosphate response factor, PHR1, prioritizing phosphate intake than defense. Zamioudis, et al. [122], have demonstrated the induction of the transcription factor MYB72 by rhizobacteria VOCs and photosynthetic related signals, enhancing iron-assimilation and inducing systematic resistance in plant. MYB72 has also been reported to regulate coumarin scopoletin root exudate, which is an iron-mobilizing phenolic compound that shapes root microbiota and improves plant growth and resistance [123]. Root exudates composition, such as carbohydrates, amino acids, and organic compounds (e.g. coumarin, rosmarinic acid), is genotype and environmentally dependent and shape the root microbiome [118,124]. However, little is known about the role of root microbiota, soil physicochemical composition and the source of nitrogen (ammonium and nitrate), and their role in the plant immune response. In this regard, future studies should address the study of defense responses in combination with the study of microbial populations in roots and the nutrient compositions of soils. Our early results carried out on the L*er*/Kas-2 NIL line and in another incompatible hybrid between accessions Uk-1 and Uk-3 [96], are suggesting a key role of the plant mineral nutrition on the modulation of the plant autoimmunity (unpublished data). For instance, we have determined that an increase on ammonium in soil or by irrigating with high-ammonium media, suppress highly the HI phenotype. Actually, a validation of endophytic plant-growth promoting bacteria PGPB), isolated from a local population of A. thaliana (Natural Park of Collserola, Barcelona, Spain), is on course. Also, we are determining the levels and the types of polyamines that PGPB produces and their effect on the plant resistance against biotic and abiotic stress.

Conclusion

The model plant of *A. thaliana* is useful for the research in plant biology and genetics. Its worldwide distribution, adaptation to the diverse environmental niches and local pathogens, through natural variation mechanism, are highly attractive for basic plant research in abiotic and biotic stress. The small genome, plantlet size, and autogamous style of reproduction are one more of its advantages when QTL mapping and GWAS are performed with the aim to discover new genes behind physiological processes and immune response. The isolation between accessions and the fixation of new genetic variants which are thought to provide, or

not, some advantage can imply unexpected deleterious effects. For instance, deleterious epistasic interaction between immune receptors and receptors like kinases that produce self-immune activation can be considered as an incipient mechanism of speciation according to the Bateson-Dobzhansky-Muller of reproductive isolation. We have described some of the genetics tools that can be used to suppress the occurrence of hybrid incompatibilities, restore hybrid normal growth and fitness. Random mutagenesis, by using chemical and physical agents, or by gene editing techniques such as, the CRISPR/Cas-9, had demonstrated their usefulness. These techniques, coupled to next generation sequencing, can help to identify non-synonym point mutation casual for the suppression of the autoimmunity and allows the study of the molecular mechanisms taking place during the plant-pathogen recognition and immune signaling. This is quite interesting in regard for plant breeders, because hybrid incompatibilities caused by immune-incompatible interactions are not exclusive to Arabidopsis and can be found in many crop-plant species (e.g. lettuce, rice, and wheat, among others).

Finally, the study of the plant-microbe molecular interaction is an emerging field in plant research. With the development of new strategies for the plant-associated microbiota isolation, the global sequencing techniques applied in genomics and transcriptomics, as well metagenomics analysis is providing a huge information of how plant basic mechanisms of nutrition, a biotic and biotic stress are shaped by soil microbiota. Furthermore, the topic of microbe-delivered polyamines on the plant immune response is not yet investigated and some early results are suggesting a positive effect on the pathogen triggered immunity acting as a priming molecule against a broad range of stress that plants suffer in crop fields [64,125].

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Chapter 4. *Trypanosoma cruzi* Infection Diagnosis: New Insights, Challenges and Perspectives

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Abstract

Chagas disease (CD), caused by the protozoan *Trypanosoma cruzi*, is endemic in Latin America and due to human migration it has become and emergent health problem in non-endemic countries. CD diagnosis is complex and no single test is considered the reference standard. After assessing a range of techniques to diagnose CD, we propose cost-effective diagnostic algorithms for each stage of the disease and evaluate different approaches to *T. cruzi* characterization. The results of the studies summarized here indicate that a single serological test with a high-performance technique can improve diagnosis of chronic cases, whereas congenital infections can be satisfactorily diagnosed using fewer tests than current strategies. The application of both proposals would entail important savings for health institutions. Additionally, a fully automated molecular methodology was satisfactorily evaluated and could help to extend the routine use of PCR for CD diagnosis among hospitals. The assessment of different typing methods for *T. cruzi* characterization also provided useful results, although a single optimal method could not be established for all cases.

Keywords: Chagas disease; Serological diagnosis; Molecular diagnosis; Molecular characterization

Abbreviations: qPCR: quantitative real-time PCR; ELISAc: conventional ELISA; ELISAr: recombinant ELISA; WB: Western blotting; TP: True positive; TN: True negative; FP: False positive; FN: False negative; S/CO signal-to-cut-off value; PAHO: Pan American Health Organization; IAC: Internal Amplification Control; Ct; Cycle threshold; SatDNA: satellite DNA, kDNA: kinetoplastid DNA; EB: EDTA-blood; GEB: Guanidine EDTA-blood; rs: Spearman rank correlation coefficient, K: Kappa coefficient; MTq-PCR: Multiplex qPCR using Taqman probes.

Introduction

Chagas disease (CD) is a parasitic infection caused by the kinetoplastid protozoan *Trypanosoma cruzi* Chagas, 1909 [1]. The disease is endemic in 21 countries of Latin America with around six million people affected [2]. Migratory flows have spread CD to non-endemic areas, especially in the United States and Europe, and it is now a global public health problem [3]. Spain is the European country with the largest number of immigrants from Latin America [4].

In endemic areas, the parasite is mainly transmitted by blood-sucking triatomine bugs [5]. The infection may also occur in both endemic and non-endemic areas through congenital transmission, blood transfusion, organ transplant, and laboratory accidents [6]. In non-endemic countries, mother-to-child transmission is the main route of infection and represents an important healthcare challenge [7,8]. Vertical transmission can occur at all stages of CD [9], in any pregnancy of an infected woman and over successive generations [10]. Congenital transmission occurs mainly by the haematogenous transplacental route [11,12].

T. cruzi has great genetic diversity and its natural populations are currently divided into six genetic subdivisions, known as discrete typing units (DTUs): TcI-TcVI [13,14]. Another DTU, first isolated from bats in Brazil, has been designated as the Tcbat genotype [15]. The concept of DTUs refers to sets of stocks genetically closer to each other than to any other stock and identifiable by common markers [13,16]. DTUs have variable distribution among endemic regions and in transmission cycles [14].

CD occurs in two stages: (i) the acute phase, in most cases without symptoms or with non-specific manifestations and characterized by a high parasitic burden in the blood, and (ii) the chronic phase, associated with cardiac and/or gastrointestinal disorders and low and intermittent parasitemias. In the chronic indeterminate phase of the infection most patients remain asymptomatic all their lives [17,18].

In Spain and other non-endemic areas, the acute phase is mainly due to congenital transmission and the chronic phase is observed in migrants infected in their country of origin. The stage of the disease conditions the choice of diagnostic method. Parasitological and molecular tests are the most suitable for the acute phase and serological for the chronic stage [19]. In conventional serological techniques the whole parasite or soluble or purified parasite extracts are used as the antigen, whereas non-conventional tests are based on recombinant antigens [20].

Although screening for CD and early diagnosis are cost-effective, a large number of patients are still diagnosed late or not at all in both endemic and non-endemic countries [21]. Thus, it is of great importance to establish a more effective diagnostic strategy to deal with *T. cruzi* infection [22].

T. cruzi diagnosis is often complex and no single test is considered the reference standard [20]. Current protocols are costly as they involve the analysis of a large number of samples by several serological tests. Serological methods, many of them commercially available, are widely used, especially in the chronic phase [23,24], but are limited because of (i) a high persistence of positive results in chronic patients after treatment [25], (ii) the transmission of passive maternal antibodies to the newborn [11,26], and (iii) the possibility of cross-reactivity with other trypanosomatids, such as *Leishmania* spp. [27]. A new generation of potentially more accurate tests has been developed but their usefulness in different population groups, infection phases and disease follow-up is not yet well established [28]. They involve the use of large mixtures of recombinant antigens combined with efficient detection systems. Automation, rapidity and high performance are other advantages of these new tests [28].

On the other hand, molecular methods, especially the polymerase chain reaction (PCR), are effective for the early diagnosis of congenital infection due to their high sensitivity [29]. They are also useful in the chronic phase to detect therapeutic failure in treated patients and parasite reactivation in immunosuppressed individuals [30]. Additionally, quantitative real-time PCR (qPCR) enables quantification of the amplification product. However, molecular approaches have some disadvantages, including less sensibility in chronic patients with low parasitic loads and a lack of consensus among laboratories in PCR strategies, which makes it difficult to compare the results obtained [31]. The recent introduction of new molecular *T. cruzi* diagnostic tools on the market could greatly improve CD diagnosis and lead to the standardization of protocols [32,33].

Finally, molecular characterization studies of *T. cruzi* have sought to identify associations between DTUs and the clinical development of CD [34]. There are many molecular markers but no agreement on the use of a specific protocol [35]. The genetic diversity of the parasite could play a key role in the management of CD and must be taken into account when developing diagnostic tests [36].

The wide diversity in the available diagnostic methods and lack of consensus in their implementation prompted our group to look more closely at the problems of CD diagnosis and the molecular characterization of *T. cruzi*. Accordingly, the following objectives were defined: (i) to assess and compare different diagnostic techniques, both serological and molecular, for the different stages of CD infection; (ii) to establish cost-effective diagnostic algorithms for each stage; and (iii) to evaluate different approaches for the characterization of *T. cruzi* genotypes in adults and newborns.

Serological Diagnosis

According to the World Health Organization (WHO) recommendations [20], chronic CD serological diagnosis

requires positive results in at least two traditional tests, selectable from the enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT), and indirect haemagglutination (IHA). In congenital cases, serological tests should be performed in infants aged >8 months to avoid the detection of maternal anti-*T. cruzi* IgG antibodies [26]. The option of performing the test later, at >10 months, has been recently proposed by the WHO Technical Group [37]. The study of IgG kinetics can be useful in the diagnosis of congenital infection [18], as well as parasitological methods and PCR in the first weeks of life [38-40]. However, direct techniques are not always effective for an early diagnosis of congenital *T. cruzi* infection and, in these cases, serology becomes necessary. Regarding IgM antibodies, their use as a diagnostic tool for congenital CD is limited in that the presence of other antibodies such as rheumatoid factor may produce false positive results [41].

Preliminary Study

A preliminary study to assess the usefulness of four serological tests for general CD diagnosis, without specifying the stage of the disease, was carried out. A total of 247 sera from patients suspected of having CD attending the Hospital de la Santa Creu i Sant Pau of Barcelona (Spain) during January 2009-December 2012 were analyzed by the following methods: (i) IFAT with *T. cruzi* epimastigotes used as the antigen (*Trypanosoma cruzi* IFA test system, Trinity Biotech, Bray Country, Wicklow, Ireland). Double dilutions of sera from 1:20 to 1:5120 were used, and a dilution of ≥1:40 were established as the cut-off. (ii) An *in house* ELISA with sonicated epimastigotes of *T. cruzi* as the antigen (ELISAc) [42]. The reaction was quantified as units (U) and the cut-off was established at 20 U. (iii) Recombinant ELISA (ELISAr) using the TcF antigen (BioELISA Chagas, Biokit, Lliçà d'Amunt, Spain). The reaction was measured in absorbance and the signal-to-cut-off value (S/CO) was ≥1 with a gray-zone from ≥0.9 to <1. (iv) A chemiluminescent microparticle immunoassay (CMIA) with the recombinant antigens FP3, FP6, FP10, and TcF (Architect Chagas, Abbott Laboratories, Wiesbaden, Germany). The reaction was measured in relative light units and the S/CO was ≥1 with a gray-zone from ≥0.8 to <1.

A serum was considered as definitely positive when at least three of the four tests yielded a positive result and the same criterion were used for the negatives. Sera were considered doubtful when only two out of the four tests produced a coincident result. For their classification, doubtful sera were analyzed by a fifth technique, an *in house* Western blotting (WB) based on lysate *T. cruzi* epimastigotes [43]. The antigenic bands of the *T. cruzi* profile are 28, 32, 38, 39, 40, and 48 KDa. A serum was considered positive when at least five out of the six bands of the pattern were recognized. The final interpretation of the results was considered as the reference standard for the subsequent calculations. Fifteen out of the 247 sera produced doubtful results (Table 1) and were excluded from the calculations.

Case	IFAT (dil.) ≥1:40	ELISAc (U) ≥20	ELISAr (S/CO) ≥1	CMIA (S/CO) ≥1	WB (bands) ≥5/6	Age
1	P (1:40)	P (53)	N (0.06)	N (0.31)	N	Adult
2	P (1:160)	P (31)	N (0.58)	N (0.08)	N	Adult
3	P (1:40)	P (29)	N (0.11)	N (0.08)	N	Adult
4	P (1:40)	P (21)	N (0.57)	N (0.04)	N	Adult
5	P (1:40)	P (28)	N (0.64)	N (0.08)	N	Adult
6	P (1:40)	P (30)	N (0.09)	N (0.32)	N	Newborn
7	P (1:40)	N (11)	P (1.74)	N (0.24)	N	Adult
8	P (1:80)	N (13)	P (1.50)	GZ (0.87)	N	Newborn
9	P (1:40)	N (16)	N (0.38)	P (5.23)	N	Newborn
10	P (1:160)	N (17)	N (0.17)	P (3.00)	N	Newborn
11	N (1:20)	P (21)	N (0.61)	P (3.96)	N	Newborn
12	N (1:20)	P (26)	N (0.76)	P (4.17)	N	Newborn
13	N (1:20)	P (27)	N (0.88)	P (4.45)	N	Newborn
14	N (1:20)	P (32)	N (0.87)	GZ (0.87)	N	Adult
15	N (1:20)	N (17)	P (1.26)	P (1.44)	N	Newborn

Table 1: Results produced by the doubtful sera (n = 15).

The results obtained in each serum and test were compared to the final interpretation of results for their classification as true positives (TP), true negatives (TN), false positives (FP), or false negatives (FN). Based on this information, measures of diagnostic accuracy were calculated for each test.

Tests based on conventional antigens, IFAT and ELISAc, shared the largest number of positive sera (n=6), whereas recombinant tests, ELISAr and CMIA, gave the highest number of coincident negative results (n=6). All the doubtful sera tested negative by WB and were probably the result of FP reactions in the evaluated techniques. Conventional tests produced the highest number of positive reactions in the doubtful sera but most were close to the cut-off. On the other hand, all CMIA positive reactions in the doubtful sera came from children less than one year of age from chagasic mothers and could be attributed to the presence of maternal antibodies in the newborns.

The 232 samples remaining after the exclusion of the doubtful sera are shown in Table 2A. A review of the patient medical histories revealed that six sera classified as FP in the CMIA were from newborns. In these cases, serology values decreased over time, indicating the presence of passive antibodies. CMIA detected maternal antibodies for longer than the other tests, resulting in six positive sera improperly classified as FP. These samples were also excluded from the calculations, leaving a final panel of 226 sera (Table 2B).

A

	IFAT	ELISAc	ELISAr	CMIA
VP	145	146	145	146
FP	7	4	1	7*
VN	79	82	85	79
FN	1	0	1	0
	232	232	232	232

B

	IFAT	ELISAc	ELISAr	CMIA
VP	145	146	145	146
FP	7	4	1	1
VN	73	76	79	79
FN	1	0	1	0
	226	226	226	226

Table 2: Summary of true and false positives and negatives for the four serological tests assessed. A) Results obtained after the exclusion of the 15 doubtful sera (n = 232). B) Results obtained after the exclusion of the 15 doubtful sera and the six sera incorrectly classified as false positives by CMIA (n = 226). * Six sera from newborns.

Measures of diagnostic accuracy for each test are shown in Table 3. ELISAc and CMIA showed the highest sensitivity, whereas the highest specificity was obtained with ELISAr and CMIA. CMIA also scored highest for accuracy and was therefore, the test with the best overall performance. These results prompted us to assess this new generation technique in two further studies, examining its diagnostic performance for both chronic and congenital CD.

Measure	IFAT	ELISAc	ELISAr	CMIA
Sensitivity 95% CI	99.32 97.63-100	100 99.66-100	99.32 97.63-100	100 99.66-100
Specificity 95% CI	91.25 84.43-98.07	95 89.6-100	98.75 95.69-100	98.75 95.69-100
Accuracy 95% CI	96.46 93.83-99.09	98.23 96.29-100	99.12 97.67-100	99.56 98.47-100

Table 3: Measures of diagnostic accuracy of the serological tests assessed (n = 226).

Chronic CD Serological Diagnosis

The overall accuracy of Architect Chagas as a single test for the diagnosis of chronic CD was assessed in a study of 315 serum samples collected from adults admitted to the Hospital de la Santa Creu i Sant Pau from January 2009 to December 2012 [28].

Samples were classified into four panels: Panel I (n=107): samples from chronic seropositive patients from CD endemic countries with coincident positive results by two ELISAs (ELISAc and ELISAr). Panel II (n = 125): samples from non-chagasic individuals from CD endemic (n=64) and non-endemic countries (n=61) with coincident negative results by ELISAc and ELISAr. Panel III (n=12): samples from individuals from endemic CD countries with discordant serological results by ELISAc and ELISAr and tested by WB for a final diagnosis (11 were considered negative and one positive). Panel IV (n=71): samples from patients with other infectious diseases to evaluate cross-reactions (eight *Leishmania*sis, seven toxoplasmosis, six amebic hepatic abscess, three malaria, six strongyloidiasis, one visceral larva migrans, three cytomegalovirus, seven human immunodeficiency virus, fur parvovirus B19, five Epstein-Barr virus, five hepatitis B virus, two hepatitis C virus, nine syphilis, and five Lyme borreliosis).

All samples were tested by the CMIA Architect Chagas assay according to the manufacturer's instructions and the results obtained are shown in Figure 1.

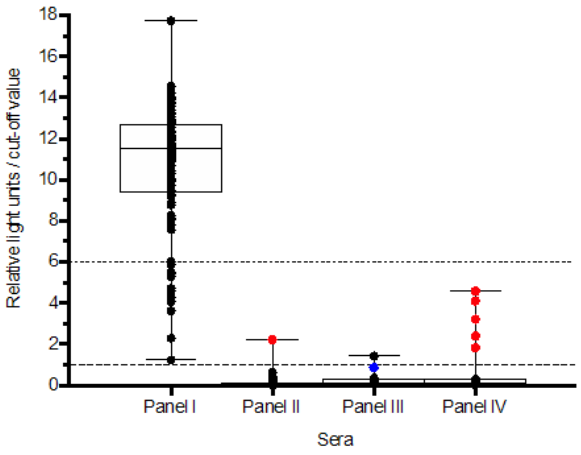

Figure 1: Overall serum value distribution of CMIA Architect Chagas. Black circles represent TP and TN results, red circles indicate FP results, and blue circles show results in the gray-zone. The dashed line represents the cut-off value established by the manufacturer (1 S/CO) and the dotted line indicates the point of 6 S/CO on the y-axis. In the box and whiskers plot, the median is shown by the line that cuts through the box, the upper edge of the box represents the 25th percentile, the lower edge represents the 75th percentile and the whiskers extend down to the minimum value and up to the maximum value.

The test showed a high sensitivity (100%) and specificity (97.6%) with only six FP, five of which were due to cross-reactions in sera from *Leishmania*sis patients (panel IV). The S/CO of these FPs varied from 1.83 to 4.57 (Figure 1). The other FP observed came from panel II and belonged to a patient with *Leishmania*sis ruled-out and without information of other possible pathologies. The test also gave a result in the gray-zone in panel III in a serum previously characterized as negative by WB. No false negative results (FN) for Architect Chagas were observed. Most (87%) TP sera (n = 108: 107 from panel I and one from panel III) gave values >6 S/CO.

The high sensitivity of the method probably comes from the four recombinant proteins used as the antigen, which in aggregate represent 14 different antigenic regions present throughout the life cycle of *T. cruzi* [44-46]. This, coupled with the use of chemiluminescence as the detection system, makes Architect Chagas a high-performance technique. Other authors have reported sensitivity of around 100% for the same method [47,48] and the Pan American Health Organization (PAHO) a slightly lower 98.9% [49]. The large number of antigens, as well as providing high sensitivity, may also explain the cross-reactions with *Leishmania* spp., which should be taken into account in areas endemic for both, visceral *Leishmania*sis and CD.

According to our results, CMIA Architect Chagas can be used as a single technique for screening in blood banks and for routine diagnosis in clinical laboratories of non-endemic

areas. Only gray-zone and positive sera with results ≤6 S/CO would need to be confirmed by a second serological assay to rule out FP sera and cross-reactions with *Leishmania* species.

Diagnosis of Congenital *Trypanosoma cruzi* Infection

The utility of five serological tests for the diagnosis and follow-up of congenital CD was assessed by the analysis of peripheral blood samples and sera from 67 newborns of Latin American Chagas-infected mothers admitted to three hospitals of Barcelona (Hospital de la Santa Creu i Sant Pau, Hospital Clínic and Hospital Sant Joan de Déu) from April 2003 to December 2015 [50]. The mothers of the newborns were diagnosed by two serological assays [20].

		Mother	0-1m	>1-6m	>9-12m	>12m	≤1m pt.	5-7m pt.	≥12m pt.
Case 1	PCR	-	-	-	Pos	-	-	-	-
	IFAT	≥1:5120	1:2560	1:640	1:640	-	1:640	-	1:80
	ELISAc	169	-	76	-	-	-	-	42
	ELISAr	8.39	8.52	6.82	6.67	-	8.70	-	1.63
	CMIA	12.31	-	7.97	-	-	-	-	3.57
	WB	6/6	-	5/6	-	-	-	-	0/6
Case 2	PCR	Pos	-	-	-	Pos	-	Neg	Neg
	IFAT	1:1280	-	-	-	1:1280	-	-	1:80
	ELISAc	200	-	-	-	162	-	116	54
	ELISAr	7.60	-	-	-	5.99	-	5.11	1.35
	CMIA	11.07	-	-	-	12.06	-	10.29	7.66
	WB	5/6	-	-	-	6/6	-	5/6	0/6
Case 3	PCR	Pos	Pos	-	-	-	-	-	Neg
	IFAT	1:2560	1:1280	-	-	-	1:320	-	-
	ELISAc	178	279	-	-	-	95	-	8
	ELISAr	5.78	5.56	-	-	-	3.52	-	0.2
	CMIA	10.9	11.22	-	-	-	5.02	-	-
	WB	5/6	5/6	-	-	-	3/6	-	-
Case 4	PCR	Pos	Neg	-	-	Pos	-	Neg	-
	IFAT	1:1280	1:1280	-	-	1:1280	-	1:320	-
	ELISAc	159	141	-	-	143	-	105	-
	ELISAr	2.65	3.1	-	-	6.41	-	5.77	-
	CMIA	12.81	-	-	-	11.64	-	7.21	-
	WB	5/6	-	-	-	5/6	-	5/6	-

Table 4: Results obtained in the congenitally infected newborns (n = 4).
m: months; pt: post-treatment.

Samples were classified into five groups according to the age of the infant when the sample was collected: birth-1 month, >1-6 months, >6-9 months, >9-12 months, and >12 months. Sera were tested by five serological assays: IFAT, ELISAc, ELISAr, CMIA, and WB. In the case of WB, a serum was considered positive when at least two bands of the pattern were recognized and also when a single band matched the intensity of the same band in the positive control. Peripheral blood samples were analyzed by qPCR [51], after DNA extraction with the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. RNAse P human gene (Life Technologies, Austin, TX) was included as an internal control of amplification (IAC). A sample result was considered valid when the RNAse P was efficiently amplified and positive when the cycle threshold (Ct) was ≤40 in at least one of the

three replicates.

An infant was considered infected with *T. cruzi* when a positive qPCR was obtained and/or the level of IgG antibodies was maintained during the first year of life, as determined by at least two serological tests. Based on these criteria, four out of the 67 infants were diagnosed as congenitally infected by *T. cruzi*. The remaining 63 newborns were considered not infected due to negative qPCR and a drop in IgG antibodies.

The results obtained in the four congenitally infected newborns are shown in Table 4. Positive results were obtained by all serological techniques performed and an antibody decline was only detected after treatment. Furthermore, the PCR was positive at some moment in the follow-up and it became negative after treatment in all tested cases. In case 4, a negative PCR result was obtained at birth and the infection was confirmed after one year, which could be explained by a late infection during delivery.

In non-infected infants, from 12 months onwards all serological tests produced negative results in all analyzed samples, with the exception of the CMIA Architect Chagas (one gray-zone sample detected) (Figure 2). Notably, despite a declining trend in passive antibodies, none of the serological assay results seroreverted in any of the non-chagasic infants in the period >9-12 months, when maternal antibodies should have disappeared completely and a positive serology indicates a congenital infection [26].

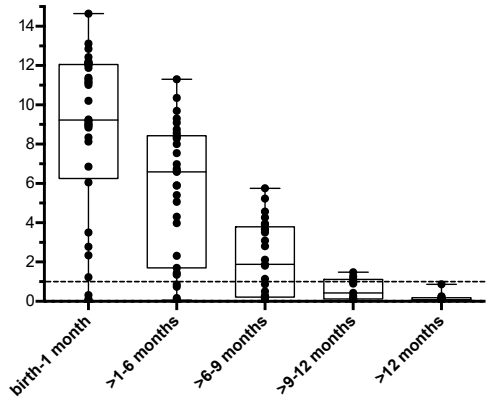

Figure 2: Results obtained by CMIA Architect Chagas during the five follow-up periods of non-infected infants. The dashed line represents the cut-off value established by the manufacturer (1 S/CO). In the box and whiskers plot, the median is shown by the line that cuts through the box, the upper edge of the box represents the 25th percentile, the lower edge represents the 75th percentile and the whiskers extend down to the minimum value and up to the maximum value.

According to our results, Architect Chagas is the most optimal for chronic CD diagnosis of all the studied tests. However, precisely due to its high sensitivity, it is not the best option for the diagnosis of congenital infection, at least when only on test is carried out at nine months of age with a cut-off ≥1. Architect Chagas detects maternal IgG antibodies in the serology of the newborn for a longer period, thus delaying the negativization of passive antibodies and making it difficult to diagnose or rule out the disease at an early stage. However, it should be emphasized that in non-infected infants of >6-9 months, at which point residual maternal antibodies were still detected, all samples yielded results <6 S/CO by Architect Chagas. In the case of infected newborns, results for this period are not available but in two infants of >12 months, the results were far higher than 6. Thus, after a slight cut-off modification Architect Chagas could be very useful in congenital CD diagnosis with as well as in chronic CD, but further studies are needed to confirm these results.

Protocols and Cost-Effectiveness

There is evidence that the global financial crisis and austerity measures have significantly affected the management of CD in Europe [52]. Although *T. cruzi* infection has become a real problem for countries that receive migrants from endemic areas [3,53], not all European states have programs to monitor the disease and there is a clear need for a common intervention program [52,54,55].

In 2002, the WHO established the criteria for CD diagnosis [20]. Today, more than a decade later, and despite the availability of new and potentially more efficient methodologies, the same recommendations are in place. Recently, the PAHO published an update of the guidelines for CD diagnosis and treatment, but it continues to recommend the old diagnostic standard to confirm a suspicious case of CD, i.e. the coincident result of two serological tests [49].

In this context, on the basis of the results we obtained in chronic CD patients, we propose the application of a high-performance technique, CMIA Architect Chagas, as an alternative for the serological diagnosis of chronic CD. The test has enough discriminatory power to correctly classify negative samples and the application of a second serological technique would only be necessary in samples with results in the gray-zone and positive values ≤6, which represented 6.3% of the samples analyzed in our study. The application of this proposal would result in significant savings in the management and control of CD. In our hospital, the savings would be more than €4,000/year compared to the cost of performing two tests for all sera, as recommended by the WHO [20].

In a posterior published research article, Pérez-Ayala,

et al. [56] proposes Architect Chagas as a single test for the diagnosis of chronic CD using a cut-off ≥3.80 S/CO. According to the authors, only positive results with an S/CO from 0.80 to 3.80 need to be confirmed by a different diagnostic test or the provision of an additional sample. Although Pérez-Ayala, et al. [54] analyzed a far larger number of samples than we did, they did not include patients with other infectious diseases such as *Leishmania*sis to evaluate cross-reactions. In our study, two out of the six FP sera produced values higher than 3.80, which might explain why the cut-off value they propose is slightly lower. In fact, the authors highlight the impossibility of evaluating cross-reactions as the main limitation of their study and recommend the use of an S/CO ≥3.80 in immigrants without clinical suspicion of *Leishmania*sis. However, patients infected with *Leishmania* spp. do not always show symptoms and asymptomatic cases remain a major challenge for diagnosis [57]. Furthermore, mixed infections of *T. cruzi-Leishmania* spp. can also occur and in fact have already been reported in countries such as Bolivia and Argentina [58,59].

In a more recent study conducted in Italy, Antinori, et al. [60] reported 100% sensitivity and 99.8% specificity for Architect Chagas. The authors agreed with our proposed strategy and, in their case, 10% of the global samples had to be confirmed by a second serological test.

Screening programs for Chagas disease in pregnant Latin American women and their children are still uncommon in non-endemic areas [61,62]. Only four European regions have implemented official prevention strategies to avoid the transmission of congenital Chagas disease: Tuscany in Italy [63] and Catalonia [64,65], Galicia [66], and Valencia [67] in Spain. The diversity of procedures used in control programs is extremely high, which emphasizes the need for a common strategy suitable for all regions.

In order to improve congenital *T. cruzi* infection diagnosis and according with the results obtained in newborns, we propose a cost-effective strategy that reduces the number of tests in the algorithms implemented in European regions and entails significant cost savings (Figure 3). In this proposal, a parasitological test is performed at birth because contact with the mother and newborn after post-delivery discharge is frequently lost [61]. If this is not possible, the protocol could start one month after birth with a parasitological test and/or a PCR. If negative, a serological test would be carried out at nine months, which if positive, would be followed by another at around 12 months for confirmation.

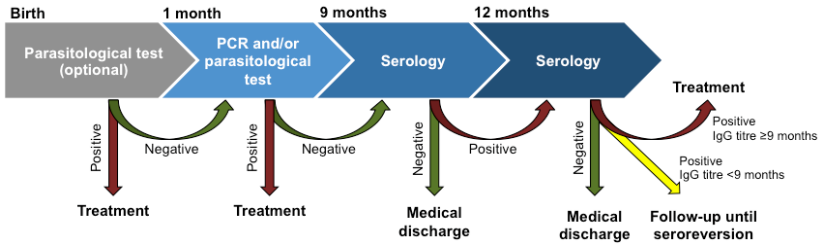

Figure 3: Diagram for congenital *T. cruzi* infection diagnosis.

The high *T. cruzi* burden in the first month of life [11,29,68] facilitates detection and test results can be considered as TP because a possible transmission of *T. cruzi* DNA from mother to fetus at birth would not be detected. The first serology is proposed at nine months because at birth positive results are due to the presence of passive maternal antibodies (97.4% of positivity in the first samples in our study). The real value of serology at birth lies in observing the basal level of antibodies for comparison with the serology at nine months to detect a possible drop in IgG antibodies and consequently exclude *T. cruzi* infection. However, this option entails high costs because of the large number of infants requiring a double serology determination. By applying the first serology at nine months, most infants would give negative results and fewer cases would require a follow-up at 12 months.

Our proposal has contributed to the improvement of the screening programme for preventing congenital transmission in Catalonia implemented in 2010 by the Catalonian Health Department [64], which has been updated by the inclusion of a PCR at one month of age and a serology follow-up at 9-12 months [65].

Molecular Diagnosis

Molecular methods, particularly PCR, represent an alternative to parasitological diagnosis, affording high sensitivity and not requiring skilled personnel for *T. cruzi* identification [18,51,69]. The qPCR is being increasingly used as a diagnostic tool in laboratories, and numerous strategies have already been developed [51,70-73]. In the

case of congenital infection, molecular tools may improve early diagnosis [38-40,74] but are not recommended until one month after birth, when the parasite burden is high [11,29,68] and also to avoid FPs results arising from parasite DNA transmission from mother to fetus [11].

The recent development of automated DNA extraction methodologies coupled to PCR systems is an important step toward protocol standardization, the main challenge in *T. cruzi* molecular diagnosis. In contrast with traditional and manual DNA extraction kits, which commonly used silica columns, automated extraction systems are usually based on magnetic separation. In reference to the subsequent DNA amplification by PCR, the most widely used molecular targets for *T. cruzi* diagnosis are the nuclear repetitive sequence of the satellite DNA (SatDNA) [51,75,76] and the mini-circle of the kinetoplastid DNA (kDNA) [73,77-80]. Both sequences are represented in a high number of copies per genome and have been satisfactorily test by international and multicentric studies [31,81]. Advantages of these combined technologies include time saving and the prevention of possible contamination of samples and reagents by human manipulation.

We therefore assessed the usefulness of an automated DNA extraction system based on magnetic particles (EZ1 Virus Mini Kit v2.0, Qiagen) combined with a commercially available qPCR assay targeting the satellite DNA (SatDNA) of *T. cruzi* (RealCycler CHAG, Progenie Molecular), a methodology used for routine diagnosis in our hospital. It was compared with a well-known strategy combining a commercial DNA isolation kit based on silica columns (High Pure PCR Template Preparation Kit, Roche Diagnostics) with an *in house* qPCR also targeting the SatDNA. We analyzed 123 blood samples collected from Hospital de la Santa Creu i Sant Pau and Hospital Clínic of Barcelona during January 2013-March 2017 [32].

Samples were classified into three panels: Panel I (n = 36): samples from non-infected individuals (19 newborns with chagasic mothers, 5 adults from CD endemic countries, and 12 adults from non-endemic countries) with coincident negative results by two serological tests. Newborns were followed-up by serology until negativization. Panel II (n=65): samples from non-treated chronic chagasic patients with coincident positive results by two serological tests. Panel III (n=22): samples experimentally spiked with cultured epimastigotes of *T. cruzi* stocks and non-spiked samples as controls.

Samples were distributed among eight different methodology protocols from A to H combining i) sample treatments: guanidine EDTA-blood (GEB) or EDTA blood (EB), ii) DNA extraction methods: Roche silica columns or Qiagen magnetic particles, and iii) qPCR approaches: *in house* or RealCycler, as shown in Table 5. Protocol A corresponds to the reference standard, previously approved by international studies [31,81], and protocol H is the fully automated methodology for its assessment (EB, Qiagen magnetic particles extraction method and RealCycler qPCR). For the *in house* qPCR a sample was considered inhibited when the internal amplification control (RNAse P human gene) gave negative results or the Ct was >29 and positive when the Ct of the target was ≤40 in at least one of the three replicates. In the case of the RealCycler CHAG, a sample was considered inhibited when the IAC gave negative results or the Ct was >35 and positive when the Ct of the target was ≤40.

	Sample treatment		DNA extraction method		qPCR		N	Positive samples	Inhibited samples
	EB	GEB	Roche silica columns	Qiagen magnetic particles	*In house*	Real Cycler			
A		X	X		X		123	72	0
B		X	X			X	123	59	0
C		X		X	X		62	8	35
D		X		X		X	62	25	17
E	X		X		X		25	11	0
F	X		X			X	25	11	0
G	X			X	X		64	15	2
H	X			X		X	64	15	0

Table 5: qPCR results of samples analyzed according to protocols used (A-H).

A high percentage of inhibition was observed in protocols C and D (Table 5), both using GEB and magnetic particles as extraction method but no inhibition appeared when the DNA was extracted within the first week after the guanidine

treatment. Thus, the inhibition is related to the time elapsed between the guanidine treatment and the DNA extraction. GEB samples are therefore unsuitable for the DNA extraction method based on magnetic particles, at least when samples are not processed immediately.

Regarding qPCR, differences were related to the number of replicates amplified in each method (three in the *in house* PCR and one in RealCycler). An equal number of replicates in both methods would probably significantly reduce the discordant results.

Results obtained by each protocol were compared with values of protocol A. For each protocol combination, Cohen's kappa coefficient (K) describing the level of concordance between two tests was calculated. The K value of 0 means no agreement and 1 perfect agreement [82]. The results of the comparison between protocols are summarized in Table 6.

Protocols	A-B	A-C	A-D	A-E	A-F	A-G	A-H
N	123	62	62	25	25	64	64
K	0.79	0.17	0.29	0.92	0.76	0.92	1
Positive samples	59	8	24	11	10	15	15
Inhibited samples	0	35	17	0	0	2	0
N excluding inhibition	123	27	45	25	25	62	64
Discordant samples excluding inhibition	13	8	10	1	3	0	0
K value excluding inhibition	0.79	0.45	0.53	0.92	0.76	1	1

Table 6: Cohen's kappa coefficient (K) results obtained for the different protocol combinations.

Protocols A and H were the only combination showing perfect agreement and no inhibition. When variations from the original protocols (A and H) were applied, the results were less convincing. The overall process therefore needs to be fined-tuned to obtain satisfactory results. In a quantitative analysis we compare the protocols by calculating a Spearman rank correlation coefficient (r_s), in which 0 means no association and 1 a very strong relationship. The r_s = 0.97, indicating high positive association between protocols. The 15 positive samples in both protocols were also analyzed by a Bland-Altman plot to quantify the agreement (mean bias = 0.12 Log_{10} par. eq./10 mL and SD = 0.43) [83]. The obtained results indicated that protocols A and H were equivalent and interchangeable [32].

The application of this totally automatic and commercial methodology (protocol H) in CD diagnosis allows results to be obtained easily and with minimum handling in just over two hours, which could encourage the implementation of *T. cruzi* PCR in more laboratories.

Trypanosoma cruzi Molecular Characterization

The genetic diversity of *T. cruzi* also plays a key role in CD diagnosis. Although the causes of the differences in the clinical presentation of CD are still not understood, the genetic variability of the parasite is closely linked to many *T. cruzi* biomedical properties such as growth ability in culture, pathogenicity in mice, transmissibility by the vector, and susceptibility to drugs [84]. The taxonomy of *T. cruzi*, always complicated, has also been hampered by the lack of standardized molecular typing methods and the use of several alternative nomenclatures [85]: biodemes [86], zymodemes [87,88], clonets [89,90], schizodemes [91], and DTUs [13,16], among others. However, a high degree of consensus has currently been reached on the nomenclature based on DTUs.

We assessed three different molecular typing approaches for the characterization of *T. cruzi* genotypes to identify DTUs in 75 peripheral blood samples from Latin American migrants who attended seven hospitals in the Barcelona area from October 2009 to February 2014 [92]. The inclusion criteria were samples with a positive result in two qPCRs, one based on SatDNA [51] and the other the conserved region of the minicircle of the *T. cruzi* kDNA [73]. The 20 samples with two positive PCRs were from Bolivian patients, comprising 14 adults; two children aged 10 and 13 years, and four newborns from chagasic mothers.

DTU characterization was performed using the following methods: i) a sequential flowchart based on multiplex qPCR using Taqman probes (MTq-PCR) [35]; ii) a conventional PCR flowchart [93,94]; and iii) SatDNA sequencing [92]. The flowcharts allowed the classification of *T. cruzi* populations at the level of a single DTU, whereas SatDNA sequencing classified samples as type I (TcI-III), type II (TcII-IV) or type I/II (TcV/VI) hybrid according to the position of a set of single nucleotide polymorphisms (SNPs) observed in the SatDNA sequence.

Ten out of the 20 samples gave positive results in the

flowcharts, 5 TcV, 3 TcII/V/VI and 2 mixed infections, one TcV plus TcII/VI and the other TcV plus TcII (Table 7). Five out of the six neonates and pediatric samples were characterized. The high parasitic load usual in congenitally infected infants facilitates DTU characterization [29,95]. All the 20 samples analyzed gave positive results with SatDNA sequencing, 19 classified as type I/II hybrid and the remaining sample as type I (undetectable results in the flowcharts). As expected, the most frequent DTU was TcV, being the most common in Bolivia, the country of origin of our patients, and predominant in peripheral blood [94,96,97]. The usual limitation of most of characterization studies is precisely their use of peripheral blood samples. Other genotypes of the parasite could be present in low parasitic loads in blood or tissues without detection [98,99].

Age group	N	SatDNA par.eq./mL	Characterized cases (PCR)	DTU	Characterized cases (SatDNA seq.)	SatDNA seq. type
Newborns (≤10 m.o.)	4	937.7-20.2	4	3:TcV 1:TcV+TcII/VI	4	4: I/II
Pediatric cases (10, 13 y. o.)	2	1.5-NQ	1	1:TcV	2	2:I/II
Chronic adults (26-43 y. o.)	14	10.5-NQ	5	1:TcV 3:TcII/V/VI 1:TcV+TcII	14	1:I 13:I/II

Table 7: DTU results obtained from the 20 samples analyzed.
NQ: non-quantifiable; m.o.: months-old; y.o.: years old; Limit of quantification (LOQ) for SatDNA qPCR: 1.53 par. eq./mL [72].

According to our results, PCR flowcharts are useful in cultured stocks and samples in the acute phase of CD but are insufficiently sensitive for samples from chronic CD patients due to their lower parasitemia. The MTq-PCR flowchart is fast, easy to interpret and avoids carry-over contamination [35]. The conventional PCR flowchart is more laborious, but it is cheaper and allows DTU identification in specimens with a lower parasitic burden than MTq-PCR. SatDNA sequence analysis enabled us to increase the number of characterized cases in chronic CD patients although it cannot discriminate between *T. cruzi* populations at the level of a single DTU. Indeed, SatDNA is a target with proven efficiency and widely used in CD diagnosis [31]. Thus, the use of the same target for the diagnosis and subsequent genotype characterization would represent a great advance. Ramírez, et al. [100] published an improved version of SatDNA sequencing that allows the identification of SatDNA TcI/III, TcII and TcIV signature patterns but is not yet capable of discriminating between the presence of hybrid lineages (TcV and VI) and the existence of mixed infections with TcI or III and TcII. Further studies in the field are needed but a door has clearly opened to the possibility of typing samples with very low parasitic loads, such as those from chronic patients. This, together with the possible association between DTUs and the clinical presentation of the disease, could bring considerable progress in the treatment of CD in the near future.

Final Considerations

More than a hundred years after the discovery of *T. cruzi* as the etiological agent of CD, diagnosis of the infection and characterization of the parasite remain challengingly. Despite the implementation and commercialization of new tests, established diagnostic protocols have not changed significantly since 2002, when the WHO published their recommendations for CD diagnosis.

The studies summarized here contribute to improving the serological diagnosis of CD. Based on the results, we propose CMIA Architect Chagas as a single test for chronic cases and a lower number of tests for congenital infections with respect to the official European strategies. Our satisfactory evaluation of a fully automated molecular methodology could also lead to improvements in diagnosis, as could the assessment of different typing methods for *T. cruzi* characterization, although it was not possible to establish an optimal method for all cases. PCR flowcharts achieved higher sensitivity and are useful in cases of acute infection, with high parasitic burden, whereas SatDNA sequencing obtained higher specificity and is more suitable for chronic patients with low parasitemia.

Conclusion

i) Architect Chagas is a highly effective assay for chronic CD diagnosis, with 100% sensitivity, and allowed the correct diagnosis of most samples when applied as a single technique. In contrast, precisely because of it high sensitivity, Architect Chagas is not the most suitable technique for *T. cruzi* congenital infection when a single serology is performed at nine months.

ii) Architect Chagas can be used as a single test for chronic CD diagnosis. Only gray-zone and positive serum samples with

a result ≤6 S/CO would need to be confirmed by a second serological assay to avoid FP sera and cross-reactions with *Leishmania* species. The application of this proposal would result in important savings in the cost of CD diagnosis and therefore in the management and control of the disease.

iii) For congenital infection diagnosis, we propose a new, more cost-effective strategy with a reduced number of tests. The protocol could start at one month of age with a parasitological test and/or a PCR. If negative, a serology would be carried out at nine months, followed by a confirmatory serological testing at around 12 months of age in case of positive results. The application of this proposal entails significant cost savings.

iv) For molecular diagnosis, the fully automated and commercial protocol assessed (EB samples, EZ1 Virus Mini Kit v2.0 and the RealCycler CHAG) is a good option for routine diagnosis of *T. cruzi* infection. When variations of the original protocols were applied, less convincing results were obtained, which indicates a need to fine-tune the overall process for satisfactory results. Our findings may contribute to the standardization of protocols between laboratories and the application of qPCR in diagnostic laboratories associated with health centers.

v) Finally, in the case of *T. cruzi* characterization, PCR-based flowcharts proved very useful for DTU identification in *T. cruzi* natural populations during acute infection but are not sensitive enough for the analysis of patients with low parasitic loads. SatDNA sequence analysis cannot discriminate between *T. cruzi* populations at the level of a single DTU but it enabled us to characterize a higher number of cases in chronically infected patients. The most frequent DTU was TcV, the most common in Bolivia and predominant in peripheral blood.

Ethics Statement

All studies summarized here were approved by the ethics committees of the participant centers: Clinical Research Ethics Committee of the Hospital de la Santa Creu i Sant Pau, the Ethics Review Committee of the Hospital Clínic, and the Research Ethics Committee of the Universitat de Barcelona. All samples were anonymized before being evaluated and included in in the studies.

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Chapter 5: Nutrients, Control of Gene Expression and Metabolic Homeostasis

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Abstract

The ability to detect changes in nutrient levels and respond appropriately to such changes is essential for the proper functioning of living organisms. Adaptation to the high degree of variability in nutrient intake requires precise control of metabolic pathways. Mammals have developed different mechanisms to detect the abundance of nutrients such as sugars, lipids and amino acids and provide an integrated response. These mechanisms include the control of gene expression from transcription to translation. Frequently, alterations in these pathways underlie the onset of several metabolic pathologies such as obesity, insulin resistance, type 2 diabetes, cardiovascular diseases or cancer. In this context, the complete understanding of these mechanisms may improve our knowledge of metabolic diseases and may offer new therapeutic approaches based on nutritional interventions and individual genetic makeup. The aim of this chapter will be to provide examples about the molecular mechanisms that connect nutrients' levels, gene expression and metabolic homeostasis.

Keywords: Nutrients; Metabolic homeostasis; Obesity

Abbreviations: GCN2: General Control 2; eIF2: Eukaryotic Initiation Factor 2; WAT: White Adipose Tissue; UCP1: Uncoupling Protein 1; BAT: Brown Adipose Tissue; AARE: Amino Acid Response Element; FGF: Fibroblast Growth Factor; PPAR: Peroxisome-Proliferator-Activated Receptor; FASN: Fatty Acid Synthase,; WT: Wild Type; HSL: Hormone-Sensitive Lipase; HFD: High-Fat Diet; LPD: Low-Protein Diets; PREDIMED: Prevención Con Dieta Mediterránea; ChREBPb: Carbohydrate-Responsive Element Binding Protein; scWAT: Subcutaneous White Adipose Tissue.

Introduction

The discovery of the galactose operon in bacteria represented a key finding for the study of regulation of metabolism. That pioneering work showed how, by modifying the level of expression of genes that code for specific enzymes, in response to the presence of certain nutrients in the environment, bacteria can adapt their metabolism to meet their nutritional needs, and connected, for the first time, changes in enzyme activity with transcriptional control of gene expression [1]. It is now commonly accepted that also

in complex organisms transcriptional regulation contributes to metabolic homeostasis.

The alteration of the mechanisms controlling gene expression (from transcription to translation), may lead to the development of metabolic diseases. Thus, understanding the effect of nutrients on gene expression may improve our knowledge of metabolic diseases and may offer new therapeutic approaches based on nutritional interventions and individual genetic makeup. For instance, the risk of having a metabolic syndrome caused by a disruption of energy homeostasis is associated with overweight and obesity. This association stresses the link between lipid and glucose metabolism.

The purpose of this chapter is to summarize some of our recent results showing how transcriptional control participates in homeostatic energy balance; particularly, how the content of protein and amino acids in the diet and possibly, other bioactive compounds, modulate transcriptional activity to achieve metabolic homeostasis.

Activating Transcription Factor 4-Dependent Induction Of FGF21 During Amino Acid Deprivation

Mammals have a wide range of mechanisms to detect and respond to episodes of malnutrition. Often starvation correlates with a lack of amino acids. The starvation of amino acids initiates a cascade signal transduction that begins with the activation of GCN2 (general control 2) nonrepressible kinase, phosphorylation of eIF2 (eukaryotic initiation factor 2) and increased synthesis of ATF4 (activation transcription factor 4) [2]. The link between amino acid intake and lipid metabolism causes the availability of amino acids in the diet to alter metabolic pathways beyond protein homeostasis. The activation of GCN2 in response to a leucine deprivation in mice results in a decreased fatty acid synthase activity and lipogenic gene expression in liver, and increased mobilization of lipid stores [3]. In addition, increased expression of β-oxidation genes and decreased expression of lipogenic genes and activity of fatty acid synthase in WAT (white adipose tissue), and increased expression of UCP1(uncoupling protein 1) in BAT (brown adipose tissue), has been observed [4,5]. GCN2 activated by its binding to any uncharged tRNA molecule, triggers the amino acid-response signal transduction pathway [6,7]. Although global protein synthesis is reduced, the translation of a group of mRNA species is increased as a part of this response. Among these is ATF4 [8,9] a transcription factor that binds to CARE, (C/EBP (CCAAT/enhancer-binding protein)/ATF-response element); also named AARE (amino acid response element) and modulates a wide spectrum of genes involved in the adaptation to dietary stress [10]. Food deprivation reduces free intracellular amino acid, and increases eIF2α phosphorylation and ATF4 mRNA levels in skeletal muscle [11]. FGF (fibroblast growth factor) 21 is a member of the FGF family, predominantly produced by the liver, but also by other tissues such as WAT, BAT, skeletal muscle and pancreatic β- cells [12-15]. FGF21 expression in liver is under tight control by PPAR (peroxisome-proliferator-activated receptor) α [16-19], it is induced in the liver during fasting and its expression induces a metabolic state that mimics long-term fasting. Thus, FGF21 is critical for the induction of hepatic fat oxidation, ketogenesis and gluconeogenesis, which are metabolic processes critical for the adaptive metabolic response to starvation [20].

We have shown that the hormone FGF21 is induced by amino acid deprivation both in mice liver and cultured HepG2 cells. We have identified the human FGF21 gene as a target gene for ATF4 and we have localized two evolutionary conserved ATF4-binding sequence in the 5' regulatory region of the human FGF21 gene. These sequences are responsible for the ATF4- dependent transcriptional activation of this gene (Figure 1) [21]. The ATF4-dependent increase in FGF21 expression has been confirmed in mice with autophagy deficiency in skeletal muscle and in liver [22]. Interestingly, these mice are protected from diet-induced obesity (DIO) and insulin resistance. These results add FGF21 gene induction to the transcriptional program initiated by increased levels of ATF4 and offer a new mechanism for the induction of the FGF21 gene expression under nutrient deprivation

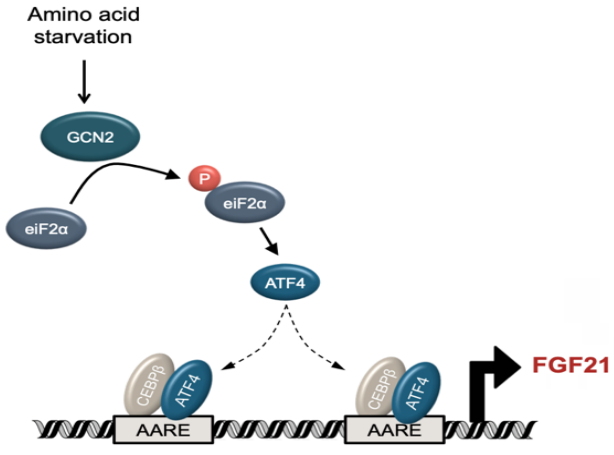

Figure 1: The starvation of amino acids initiates a cascade signal transduction that begins with the activation of GCN2, phosphorylation of eIF2 and increased synthesis of ATF4.

FGF21 Mediates the Lipid Metabolism Response to Amino Acid Starvation

In addition to GCN2-dependent inhibition of fatty acid synthase (FASN) activity, expression of lipogenic genes in liver, and increased mobilization of lipid stores in response to leucine deprivation in mice described above, the following have been observed: increased expression of β-oxidation genes, decreased expression of lipogenic genes and activation of FASN in WAT, and increased expression of Ucp1 in BAT [4,5].

The coincidence between the metabolic response to essential amino acid deprivation and to FGF21, and the induction of FGF21 under amino acid deprivation [21] led us to consider that FGF21 could be an important mediator between amino acid deprivation and lipid metabolism in liver, WAT, and BAT.

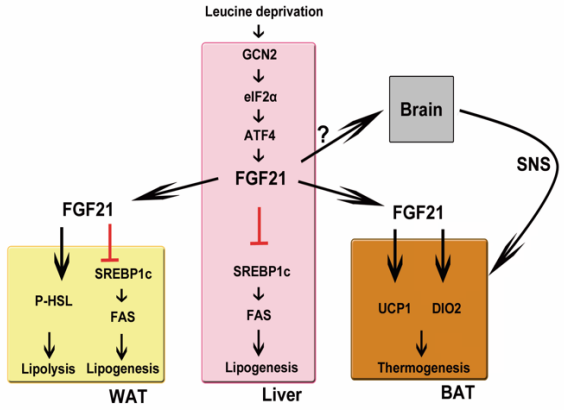

Figure 2: Working model of the FGF21 regulatory pathway under leucine deprivation.

To investigate this hypothesis, we examined the response of FGF21-deficient mice to deprivation of the essential amino acid leucine. As expected, we found a huge increase in FGF21 expression in the liver of wild-type (WT) animals, along with a repression of lipogenic genes after 7 days of leucine deprivation. In this condition, FGF21-deficient mice developed liver steatosis caused by unrepressed expression of lipogenic genes. In WAT, the expression of lipogenic genes was also repressed and the phosphorylation of hormone-sensitive lipase (HSL) was increased under leucine deprivation. The absence of leucine also induced an increase in the expression of Ucp1 and type 2 deiodinase (Dio2) in BAT. We found that all these effects in WAT and BAT were also impaired in FGF21-deficient mice. These results show the involvement of FGF21 in the regulation of lipid metabolism during amino acid starvation, thus reinforcing its important role as an endocrine factor in coordinating energy homeostasis under a variety of nutritional conditions [23] (Figure 2).

A Low-Protein Diet Induces Body Weight Loss and Browning of Subcutaneous White Adipose Tissue through Enhanced Expression of Hepatic Fibroblast Growth Factor 21 (FGF21).

Methionine-deprived mice show a phenotype comparable to that of leucine-deprivation, including resistance to a high-fat diet (HFD), improved glucose homeostasis, increased fatty acid activation and oxidation in liver, enhanced lipolysis in WAT, and increased UCP1 expression in BAT. The induction of hepatic FGF21 expression under these conditions was found to be accompanied by an increase in FGF21 protein levels in serum [24,25]. In order to facilitate the translation of these findings to humans, we focussed our work on low-protein diets (LPD) instead of amino acid-deficient diets. Protein restriction brings about weight loss and an increase in both food intake and energy expenditure [26]. Moreover, a LPD induces thermogenic markers in BAT of obese rats [27]. Serum concentrations of FGF21 in both rodents and humans increase upon exposure to an LPD, regardless of total calorie intake. This observation thus, reveals that FGF21 is likely to be involved in the metabolic response to protein-restricted diets [28].

To study the effects of a LPD on lipid metabolism, we examined the metabolic response of wild-type and FGF21 liver-specific knockout mice to a LPD (up to 5% of energy as protein). A decreased in dietary protein content induced a huge increase in FGF21 serum levels, significant weight loss, and an increase in the expression of UCP1 in the subcutaneous WAT of wild-type mice. Remarkably, no effects were observed in FGF21-deficient mice, thereby indicating that the absence of FGF21 blunts or completely blocks the response to a LPD in this mouse model. To corroborate these results in humans, we evaluated whether protein intake is associated with circulating levels of FGF21. We calculated protein intake through nutritional questionnaires and determined the serum levels of FGF21 in 78 individuals randomly selected from two nodes of the PREDIMED (Prevención con Dieta Mediterránea) trial. As with the animal model, an inverse correlation between circulating FGF21 levels and protein intake was observed [29] (Figure 3).

The data collected from humans raises the possibility of investigate the dietary modulation of circulating levels of FGF21 as an alternative approach to its pharmacological

administration.

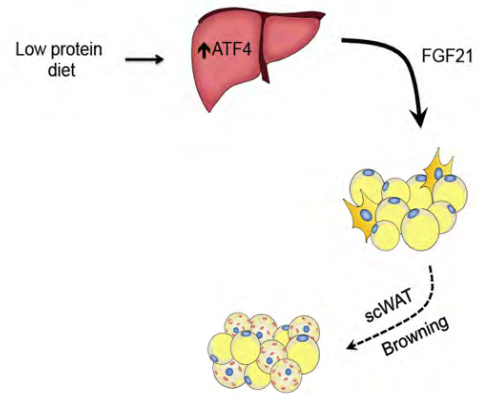

Figure 3: Molecular mechanisms by which a LPD exerts its metabolic effects through the induction of hepatic FGF21 expression and browning of scWAT.

Lyophilized Maqui (Aristotelia Chilensis) Berry Induces Browning in the Subcutaneous White Adipose Tissue and Ameliorates the Insulin Resistance in High Fat Diet-Induced Obese Mice.

Stimulation of BAT and the induction of browning in WAT as a strategy against obesity and its associated metabolic complications have generated growing interest in recent years. This interest is based on the ability of both BAT and browned-WAT to increase energy expenditure mainly through fatty acid consumption [30,31], and their pivotal role in the control of energy homeostasis in mammals [32,33]. In addition to classical brown adipocytes located in BAT, thermogenic adipocytes with similar characteristics can be found within WAT. These brite/beige adipocytes are metabolically and phenotypically similar to brown adipocytes and can actively contribute to increasing whole-body energy expenditure. Specifically, brite/beige adipocytes show a multilocular phenotype and express genes closely related to BAT metabolism (Ucp1 as a marker of its thermogenic capacity in addition to genes implied in de novo lipogenesis, fatty acid oxidation, lipolysis, etc.).

Recent evidence shows that the activation of BAT and the induction of browning in WAT can be induced by cold acclimation but also by nutritional inputs under different signaling cascades [29,31,34,35]. Regarding nutritional inputs, we recently demonstrated that low-protein diets and the cooked-tomato sauce called "sofrito" are able to induce Ucp1 expression in WAT, thus indicating a browning phenotype [35]. Other authors published that high-fat diets, bioactive compounds and prebiotics can also induce browning in WAT [36-41]. Part of the cold-induced metabolic profile in BAT is regulated by the stimulation of carbohydrate-responsive element binding protein b (ChREBPb) through the AKT2 activity [42]. Besides ChREBP, FGF21 has shown beneficial effects on glucose/lipid homeostasis and body weight control among other mechanisms by increasing energy expenditure and inducing browning and UCP1 overexpression in adipose tissues [29,43-46], as well as by promoting the insulin-dependent glucose uptake, mitochondrial biogenesis, and adiponectin secretion in adipocytes [47,48]. In this case, it has been widely demonstrated that FGF21 activity and/or signaling respond to nutritional challenges [49].

Anthocyanidin-rich berries have been proposed for the treatment and prevention of several disorders, including obesity-related metabolic disorders, but little is known about the molecular mechanisms underlying their beneficial effects. Maqui (Aristotelia chilensis) is a native Chilean berry with a unique anthocyanins profile that includes delphinidin-3-O-sambubioside-5-O-glucoside and delphinidin-3-O-sambubioside as the main phenolic compounds [50]. Besides its antioxidant activity, different preparations of maqui have shown positive effects on fasting glucose and insulin levels in humans and murine model of type 2 diabetes and obesity [51,52] and delphinidin-3-sambubioside-5-glucoside has been described as the responsible for hypoglycemic activity in *in vivo* models.

We investigated the molecular mechanisms underlying the impact of maqui on the onset and development of the obese phenotype and insulin resistance in high fat diet-induced obese mice supplemented with a lyophilized maqui berry. Maqui-dietary supplemented animals showed better insulin response and decreased weight gain but also a differential expression of genes involved in de novo lipogenesis, fatty acid oxidation, multilocular lipid droplet formation and thermogenesis in subcutaneous WAT. These changes correlated with an increased expression of Chrebpb, the sterol regulatory binding protein 1c (Srebp1c) and Cellular repressor of adenovirus early region 1A–stimulated genes 1 (Creg1) and an improvement in the FGF21 signaling. Our evidence suggests that maqui dietary supplementation activates the induction of fuel storage and thermogenesis characteristic of a brown-like phenotype in subcutaneous WAT and counteracts the unhealthy metabolic impact of an HFD. This induction constitutes a putative strategy to prevent/treat diet-induced obesity and its associated comorbidities [53] (Figure 4).

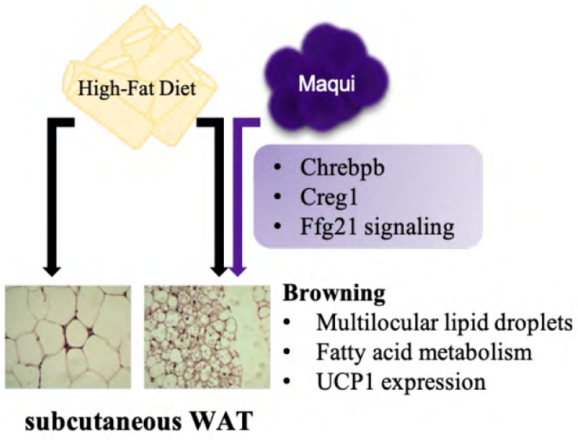

Figure 4: Maqui-dietary supplemented animals showed a differential expression of genes involved in de novo lipogenesis, fatty acid oxidation, multilocular lipid droplet formation and thermogenesis in subcutaneous white adipose tissue (scWAT).

Conclusion

In this chapter, we have presented some molecular mechanisms of diet-induced changes in gene expression, which allows the integration of nutrient signaling to metabolic homeostasis. Although not discussed in this paper, it is well-known that dysregulations on signaling transduction pathways may be the trigger of the development and progression of metabolic disorders such as obesity and type 2 diabetes, thus revealing a complicated network of regulatory mechanisms to achieve metabolic homeostasis.

Several examples, not presented here, illustrate the connection between alterations in the signaling pathways and metabolic diseases and the relevance of this knowledge for the development of efficacious therapeutic agents for the treatment of these disorders. These examples point out the importance of the knowledge/understanding of the molecular mechanisms that, through regulating gene expression, control metabolism in response to dietary inputs to design new therapeutic strategies against metabolic diseases based on nutritional interventions.

Acknowledgements

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Chapter 6: Polymeric Nanoparticles for the Treatment of Neurodegenerative Diseases: Alzheimer 's Disease and Glaucoma

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Abstract

Neurodegenerative diseases are one of the most prevalent group of pathologies affecting developed populations. It corresponds to an umbrella term for a wide variety of pathologies including Alzheimer's disease (AD) and glaucoma. AD is especially relevant since it constitutes the most common form of dementia. On the other hand, glaucoma is defined as an ocular neurodegenerative pathology. Both diseases are associated with aging and also in both of them drugs have to overcome two similar and highly restrictive barriers: the blood-brain barrier (BBB) and the blood-retinal barrier (BRB). In these barriers, tight junctions sealing endothelial cells limit the transport of a high number molecules, including a the majority of neuroprotective drugs. In this sense, drug delivery systems and especially polymeric nanoparticles, due to their non-toxicity and biodegradability, could be of great use in order to encapsulate drugs that are not able to arrive to their target site due to physiological barriers (BRB and BBB). Several strategies in order to develop biodegradable polymeric nanoparticles encapsulating neuroprotective drugs able to cross the BBB or BRB have been developed. As explained throughout the chapter, recent investigations engineering the nanoparticles by attaching peptides or antibodies in order to increase their bioavailability into the brain have been employed during the last years.

Keywords: Alzheimer's disease; Glaucoma; Nanoparticles; PLGA; Neuroprotection

Abbreviations: AD: Alzheimer's Disease; Aβ: Amyloid Beta; IOP: Increased Intraocular Pressure; BBB: Blood-Brain Barrier; BRB: Blood Retinal Barrier; NPs: Nanoparticles; APP: Amyloid Precursor Protein; CNS: Central Nervous System; PECAM1: Platelet Endothelial Cell Adhesion Molecule; JAMS: Junctional Adhesion Molecules; RMT: Receptor-Mediated Transport; CMT: Carrier-Mediated Transport; ISF: Brain Interstitial Fluid; ABC: ATP-Binding Cassette, DHA: Docosahexaenoic Acid; RPE: Retinal Pigment Epithelium; RNFL: Retinal Nerve Fiber Layer; AChE: Acetylcholine Esterase. SOD: Superoxide Dismutase

Introduction

Neurodegenerative diseases are defined as a group of pathologies that constitute the major cause of disability and premature death among elder population worldwide [1]. Among all, Alzheimer's disease (AD) is the most common form of neurodegenerative pathology [2]. The main characteristics symptoms of AD are the memory loss and cognitive decline of the patients. This disease causes neuronal cell death and is characterized by the early apparition of amyloid beta (Aβ) plaques and the neurofibrillary tangles of the hyperphosphorylated microtubule-associated protein tau [1,2].

Conversely, glaucoma is an ocular neurodegenerative disease that affects the inner layers of the eye and is the leading cause of irreversible vision loss worldwide [3]. Although increased intraocular pressure (IOP) had been postulated as the major risk in glaucoma, this disease is characterized by progressive retinal ganglion cell (RGC) death. It has been recently suggested that IOP-independent mechanisms are implicated in glaucomatous degeneration [3]. An increasing amount of similarities exists between glaucoma and AD. Both pathologies include the selective loss of neuronal populations, transsynaptic degeneration in which the disease spreads from injured neurons to connected neurons, and common mechanisms of cell injury and death [3]. Moreover, mechanisms involved in central visual system damage in glaucoma include oxidative injury and glutamate excitotoxicity similar to AD [3].

In this sense, drug delivery systems could be of great use to treat neurodegenerative diseases due to the fact that they could be designed in order to facilitate that the drugs overcome restrictive barriers such as the blood-brain barrier (BBB) in AD and the blood retinal barrier (BRB) in glaucoma, in order to arrive to the target cells. Among all, polymeric nanoparticles (NPs) had been acknowledged as a suitable vehicle to overcome these problems. PLGA have emerged as a promising polymer to be used for drug delivery since it is biocompatible, biodegradable and non-toxic. These NPs are able to encapsulate drugs and deliver them into the target site obtaining a prolonged drug release and decreasing side effects. In addition, these systems can be functionalized in order to increase the transport through specific barriers [4].

Alzheimer's Disease and the Blood-Brain Barrier

AD is the most common form of neurodegenerative disease, although its etiology remains unknown. Aβ accumulation in the brain is an early hallmark of AD widely believed to have pathogenic importance although additional amyloid precursor protein (APP)-dependent and independent cell dysfunction is increasingly suspected to be critical to the development of AD [3]. In order to arrive to the brain, the drugs have to overcome the BBB. This barrier exerts a protection for the neuronal cells and maintains the central nervous system (CNS) internal milieu, which is required for proper synaptic and neuronal functioning [5].

The BBB consists of an endothelial membrane formed by endothelial cells without fenestrations with brain microvessels that have sealed cell-to-cell contacts (tight-junctions) and is sheathed by mural vascular cells and perivascular astrocyte end-feet [5,6]. The capillaries form the majority of the brain vessel length providing around 12 m^2 of endothelial cell surface area, available for transport of solutes from the blood to the brain and vice versa. In contrast with the highly permeable systemic capillaries, brain capillaries and the tightly sealed endothelium, restricts the entry of most blood-derived molecules into the brain [5,7]. The endothelial cells and pericytes are surrounded by a basement membrane composed of collagen type IV, laminin, fibronectin, and heparin sulfate proteoglycan [6]. Tight-junctions connect endothelial cells and are formed mainly by occluding and claudin which interacts with transmembrane proteins forming a physical barrier [5,7]. Adherent junctions connecting endothelial cells involve cadherins, platelet endothelial cell adhesion molecule (PECAM1) and the junctional adhesion molecules (JAMs) JAMA, JAMB and JAMC [5,7].

Transport trough the Blood-Brain Barrier

Despite the high restrictive transport across the BBB, substances can be transported using different mechanisms (Figure 1). The more common transport routes are listed below:

Receptor-Mediated Transport (RMT): It enables trans-endothelial transport of proteins and peptides in both directions: from blood to brain (transferrin and insulin) and from brain to blood (apolipoproteins) [5]. Moreover, peptide bonds prevent larger peptides and proteins from using the RMT systems to cross the BBB [7].

Simple Diffusion: for O_2 and CO_2 and small lipophilic molecules.

Carrier-Mediated Transport (CMT): CMT systems are expressed by genes within the solute carrier transporter gene family, which comprises >300 transporter genes, encoding membrane-bound proteins that facilitate the transport of a wide array of substrates across biological membranes [7]. The CMT transporters, excitatory amino acid transporter 1 (EAAT1) and excitatory amino acid transporter 2 (EAAT2), clear neurotoxic molecules such glutamate and aspartate [5].

Ion Pumps: ion concentrations are regulated by different ion pumps such as the sodium pump (Na^+/K^+ATPase) on the abluminal membrane which regulates sodium influx into the brain interstitial fluid (ISF) in exchange for potassium [5], the luminal Na+ /H+ exchanger, the Cl^- /$HCO3^-$ exchanger, the luminal $Na^+/K^+/Cl^-$ cotransporter and the Na^+/Ca^{2+} exchanger.

ATP-Binding Cassette (ABC) active efflux transporters limit entry of drugs, xenobiotic products and drug conjugates [5]. Multiple ATP-binding cassette (ABC) proteins are expressed on the luminal, blood-facing endothelial plasma membrane of the BBB, which restricts the permeability of a large number of toxins, including therapeutic agents. Decreased expression and/or functional activity of ABC BBB transporters were reported in patients with AD and were shown to lead to accumulation of Aβ in the brain in an animal model of this disease [7].

Phosphatidylinositol-Binding Claritin Assembly Protein (PICALM)-Mediated Transcytosis and LDL Receptor-Related Protein 1 (LRP1) remove neurotoxic substances and toxic Aβ species linked to AD. However, levels of receptor for advanced glycosylation end products (RAGE) are increased in AD, which promotes increased re-entry of circulating Aβ, thereby increasing its brain levels [5].

Major Facilitator Superfamily: Docosahexaenoic acid (DHA), an essential omega-3 fatty acid, is transported into the brain by the endothelial major facilitator superfamily domain-containing protein 2a (MFSD2a). It has been reported that mice lacking Mfsd2a show brain DHA deficits and develop BBB breakdown, suggesting that MFSD2a has the dual function of transporting fatty acids into the brain and maintaining BBB integrity [7].

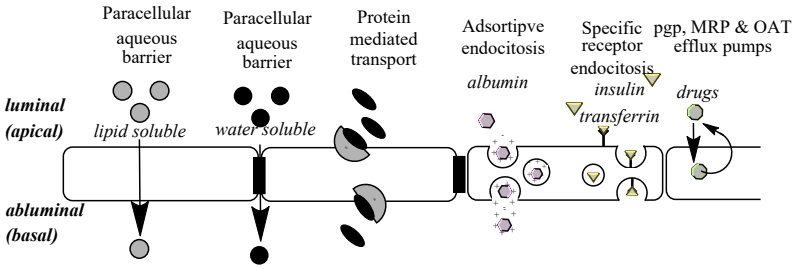

Figure 1: Key transport properties of the capillary endothelium. Modified from [5,8].

The Blood-Retinal Barrier and Glaucoma

Glaucoma is characterized by a progressive loss of RGCs, and is often associated with damage to the anterior segment and increased IOP [9].

The RGC localized in the retinal tissue are protected by BRB. The BRB, like the BBB, controls fluid and molecular movement between the ocular vascular beds and the retinal tissues and prevents leakage into the retina of macromolecules and other potentially harmful agents. This barrier plays an important role in the homeostatic regulation of the microenvironment in the retina [10]. The BRB is divided in inner and outer segments.

The inner BRB (iBRB) is mainly formed by retinal capillary endothelial cells sealed by tight junctions. Endothelial cells are surrounded by astrocytes, muller cells and pericytes thus contributing to the correct functioning of this barrier [10]. The retinal capillary endothelial cells are not fenestrated and are sealed by the tight junctions or zonula occludens of the retinal vascular endothelium [10]. The tight junctions confer highly selective barrier properties to the capillaries by restricting transport of the majority of molecules [10]. Pericytes, separated from the endothelial cells by the basal lamina, are contractile cells that regulate vascular tone, support the capillary wall, secrete extracellular material including fibronectin and possess phagocytic function. The portion of the basement membrane of the capillaries interposed between the endothelial cells and pericytes is thin, thus permitting communication between these two cell types [10]. Müller cells play a critical role in the formation and maintenance of the BRB, in the uptake of nutrients and in the disposal of metabolites under normal conditions. They are involved in the control and homeostasis of ions, signaling molecules and in the control of extracellular pH [10]. Astrocytes originate from the optic nerve and migrate to the nerve fiber layer during development. They are closely associated with the retinal vessels and help to maintain their integrity. Astrocytes are known to increase the barrier properties of the retinal vascular endothelium by enhancing the expression of the tight junctions, protein ZO-1 and modifying endothelial morphology [10].

The outer BRB (oBRB) is formed by retinal pigment epithelial cells sealed by tight junctions [10]. The retinal pigment epithelium (RPE) resting upon the underlying Bruch's membrane separates the neural retina from the

fenestrated choriocapillaris and plays an important role in transporting nutrients from the blood to the outer retina [10]. Moreover, similar to the tight junctions of the iBRB and BBB; occludin-1, claudins, and ZO-1 have been identified as the tight junctions between RPE cells. In addition, the polarized distribution of RPE membrane proteins contributes to the function of the oBRB [10].

Transport Trough the Blood-Brain Barrier

Despite the high restrictive transport through the BRB, substances can be transported using different mechanisms. The more common transport routes are listed below.

Passive Permeability: It has been observed that in the retinal vessel endothelium the increase in permeability is achieved by increasing lipophilicity or active transport [11]. It has been proposed that transendothelial transport by a system of caveolae [10].

Carrier Mediated Transporter: It facilitate the transport of a wide array of substrates across biological membranes [7].

Efflux Transporters: Efflux protein activity, both in RPE and in retinal vessels, restricts movements of certain drugs to retina. It constitutes an important protective system for the retina, but it also complicates drug delivery to posterior eye segment [11].

P-glycoprotein: expressed on the apical and basolateral RPE cell membranes has many mechanisms that contribute to trans-RPE molecular movement including the removal of unwanted molecules from the subretinal space [11].

Similarities between Glaucoma and AD

Aggregates of the hyperphosphorylated microtubule-associated protein tau in neurofibrillary tangles and neuropil threads, together with deposits of Aβ, are characteristic of AD [2].

The retina is embryologically derived from the cranial part of the neural tube, similar to the brain, and therefore, it shares many similarities. In addition, retinal thickness is decreased in AD. This fact confirms that neurodegenerative diseases may be reflected by retinal changes [2]. Glaucoma is an important potential confounder as it is a neurodegenerative disease of the retina resulting in retinal nerve fiber layer (RNFL) loss.

In addition, the prevalence of glaucoma is increased in AD patients; 25.9 %, compared to 1%–5.2% in the normal population. The reverse correlation is less distinct as some population studies of glaucoma patients show a higher risk of AD, whereas others reported no association. Possibly, risk factor for glaucoma, or AD and glaucoma share a pathophysiological process with retinal neurodegeneration as final common pathway. Two recent studies of Eraslan, et al. and Cesareo, et al. showed similar patterns of RNFL thinning, visual field loss, and optic nerve head morphology in AD and normal tension glaucoma [12,13]. Consequently, it seems very challenging to discriminate retinal changes due to AD from retinal changes due to glaucoma. Therefore, stressing the need to account for glaucoma as a contributor to retinal thickness decreases [2].

Biodegradable Nanoparticles

Advancement in the field of nanotechnology and its applications to the field of medicines and pharmaceuticals has revolutionized the 20th century [1]. The word “nano” means very small or miniature size. Nanotechnology deals with materials in ton the nanometer size of 1 to 100 nm; however, it is also inherent that these materials should display different properties such as electrical conductance chemical reactivity, magnetism, optical effects and physical strength, from bulk materials as a result of their small size. Thus, it can be used for a broad range of applications and the creation of various types of nano materials and nano devices.

As shown in the schematic diagram (Figure 2), pharmaceutical nanotechnology is divided in two basic types: nanomaterials and nanodevices. These nanomaterials can be sub classified into nanocrystalline and nanostructured materials. Nanostructured materials can be polymeric or non-polymeric. Polymeric nanostructured systems comprise nanoparticles, dendrimers, micelles and drug conjugates, among others [14].

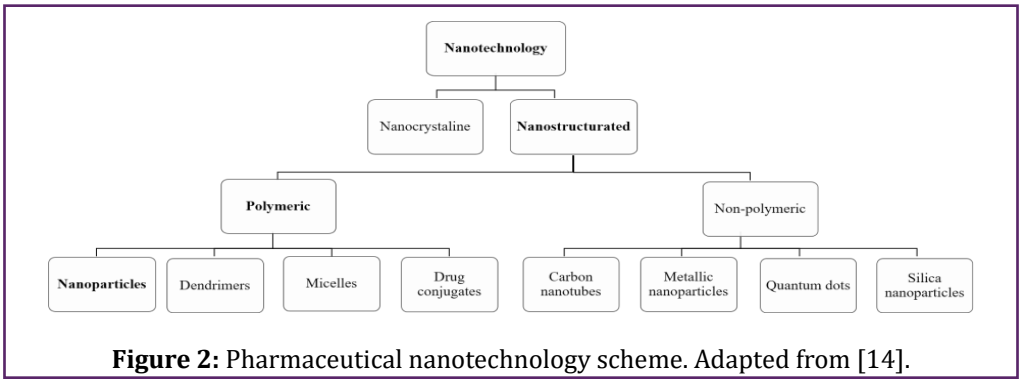

Figure 2: Pharmaceutical nanotechnology scheme. Adapted from [14].

Among the different types of NPs, PLGA nanoparticles have shown to be one of the most widely used drug delivery systems.

Polymeric Nanoparticles for AD and Glaucoma: Neuroprotection-based Strategies

Due to the high restrictive properties of BBB and BRB, the majority of large molecules do not cross these barriers. Moreover, more than 98% of all small, hydrophilic molecules cannot cross these barriers [15]. Therefore, designing strategies that aid drug passage become of crucial relevance. Among these, nanotechnology-based strategies have gained tremendous importance in order to overcome the barriers improving drugs bioavailability. These include various types of NPs for controlled drug delivery and release pertinent to various CNS conditions [16].

Among all the strategies, polymeric NPs are one of the most investigated, and they are aimed to decrease toxicity and increase safety and effectiveness of drugs [3]. Compared with other colloidal carriers, polymeric NPs increase drug stability in biological fluids. Also, their polymeric nature allows the attainment of desired properties such as controlled and sustained drug release.

Moreover, increasing reports of similarities in glaucoma and other neurodegenerative conditions have led to speculation that therapies for brain neurodegenerative disorders may also have potential as glaucoma therapies. There are several drugs that have been used for AD and for glaucoma as neuroprotection strategies encapsulated in polymeric drug delivery systems.

Curcumin

Curcumin is a yellow polyphenol found in turmeric, a widely used culinary ingredient (specially in India) that possesses anti-inflammatory properties and may show efficacy as a potential therapeutic agent in several neuro-inflammatory diseases [17]. However, this compound shows poor aqueous solubility and sub-optimal systemic absorption from the gastrointestinal tract being this the possible explanation for its failure in clinical trials for AD. To increase curcumin's bioavailability, a polymeric NPs encapsulating curcumin (NanoCurc®) were formulated. NanoCurc® are PLGA based NPs targeted with tet-1 peptide [18]. This 12-amino acid peptide has an affinity to neuronal cells and possess retrograde transportation properties [19-21]. NanoCurc™ treatment has shown to protect neuronally differentiated human SK-N-SH cells from ROS mediated insults. Moreover, in an *in vivo* mice model, NanoCurc™ PLGA NPs show to increase curcumin bioavailability in brain tissues [17]. Other authors have also produced dry curcumin NPs with a mean particle size <80 nm. These NPs were made of polyethylene-glycol-polylactic acid co-block polymer (PLGA-PEG), polyvinylpyrrolidone (PVP) as a surfactant and before lyophilization, β-cyclodextrin was added as a cryoprotectant. These freeze-dried NPs showed an increase in therapeutic efficacy in a triple transgenic mouse model (Tg2576) of AD. In addition, curcumin freeze dried NPs, produced significantly higher curcumin concentration in plasma and increased in brain curcumin amount [17]. Other successful strategies have also been employed to increase curcumin solubility and bioavailability such as the encapsulation in Poly(n-butylcyanoacrylate) (PnBCA) NPs decorated with ApoE3 ligands. They were used to exploit LDL-r-mediated transcytosis across the BBB increasing curcumin efficacy against beta amyloid induced cytotoxicity in SH-SY5Y neuroblastoma cells [22]. Moreover, Davis et al. have also demonstrated curcumin benefits in drug delivery nanosystems for glaucoma [23]. The authors show beneficial effects of curcumin in an *in vivo* rat glaucoma model observing an increase of the RGC survival [23]. Also, Cheng and colleagues prepared thermosensitive chitosan-gelatin-based hydrogel containing curcumin-loaded nanoparticles and latanoprost as a dual-drug delivery system for glaucoma treatment and they observed that curcumin could be a potential treatment for glaucoma [24].

Memantine

The main mechanism of action of memantine is believed to be the blockade of current flow through channels of N-methyl-d-aspartate (NMDA) receptors, a glutamate receptor subfamily broadly involved in brain function. Excessive activation of the NMDA receptor signaling cascade leads to excitotoxicity, wherein intracellular calcium overloads RGCs and other neurons, causing cell death through apoptosis [14]. Memantine is already marketed for its use on moderate to severe AD but it has not shown to cure the disease. In order to solve this issue, PLGA NPs are a possible solution to increase drugs therapeutic efficacy. Memantine PEG–PLGA NPs were prepared using the double emulsion-solvent evaporation method and showed a mean particle size below 200 nm and a monomodal size distribution. Memantine PEG–PLGA NPs show a slow release profile against the free drug solution, allowing to reduce drug administration frequency *in vivo*. Moreover, the authors demonstrated that nanoparticles were able to cross BBB both *in vitro* and *in vivo* and an enhanced benefit of decreasing memory impairment in comparison to the free drug solution. Histological studies confirmed that MEM–PEG–PLGA NPs reduced Aβ plaques and the associated inflammation characteristic of AD [25].

In addition, Memantine has also been assessed for glaucoma. Oral administration of memantine to non-human primates with experimentally induced glaucoma

conferred protection of RGCs. A phase III clinical trial has been completed to investigate the efficacy memantine in glaucoma neuroprotection after oral administration. However, memantine failed to reach the primary efficacy end point of reducing visual field loss in patients at a high risk of developing glaucoma [14]. In this sense, our group have successfully loaded developed a topical formulation of memantine-loaded PLGA-PEG NPs and investigated its efficacy of this formulation using a well-established glaucoma model. MEM-NPs were additionally found to be well tolerated *in vitro* (human retinoblastoma cells) and *in vivo* (Draize test). MEM-NP eye drops were found to significantly ($p < 0.0001$) reduce RGC loss. These results suggest that topical MEM-NP is neuroprotective in an experimental glaucoma model [14].

Acetylcholine Esterase Inhibitors

The clinical efficacy of Acetylcholine esterase (AChE) inhibitors for AD remains limited mainly due to poor brain translocation, which requires frequent injections, and its adverse cholinergic effects on peripheral organs.

Wilson et al. prepared PnBCA NPs encapsulating Rivastigmine and coated with Polysorbate 80 shown to be more effective in vivo for AD [26]. The same group also described a similar approach to increase the brain uptake of tacrine, another AChE inhibitor, using PnBCA nanocarriers. In this case, the use of NPs increased the tacrine brain concentration by a factor of 4 when compared with the free drug [16]. The strategy of using Polysorbate 80 for coating NPs has also been used by other authors in order to increase NPs transport trough the BBB demonstrating effective results [27].

AChE inhibitors have also shown to exert neuroprotection in glaucomatous processes. Almasieh and colleagues demonstrated that galantamine administered systemically to a rat model of glaucoma preserved microvasculature density and improved retinal blood flow in glaucomatous retinas [28]. Other authors assessed also Rivastigmine effects demonstrating that it lowers the IOP in rabbits [29]. To date, no records have been found of the use of polymeric NPs in order to deliver these compounds more effectively to the retinal tissue.

Anti-Inflammatory Drugs

Neuronal inflammation has been reported in AD [30]. This inflammation contributes to astrocytes and microglial activation increasing neuronal cell death. Therefore, a possible strategy to palliate AD could be the use of anti-inflammatory drugs such as NSAIDs. Moreover, using a mice model of human pigmentary glaucoma, some authors demonstrated the expression of the IL-18 protein and gene in the iris/ ciliary body and increased level of IL-18 protein in the aqueous humor of DBA/2J mice. This increase precedes the onset of clinical evidence of pigmentary glaucoma, implying a pathogenic role of inflammation/immunity in this disease [31].

In this sense, our group have developed Dexibuprofen loaded PLGA-PEG NPs prepared by solvent diffusion method designed to increase Dexibuprofen brain delivery reducing systemic side effects such as gastric toxicity. The NPs show to increase Dexibuprofen brain permeation coefficient. Behavioral tests performed in APPswe/PS1dE9 mice (familial AD mice model) showed that nanospheres reduce memory impairment more efficiently than the free drug. Developed nanospheres decrease brain inflammation leading to Aβ plaques reduction. According to these results, chronical oral Dexibuprofen PLGA-PEG NPs could constitute a suitable strategy for the prevention of neurodegeneration [32].

Cyclooxygenase (COX) is the rate-limiting enzyme for prostanoid synthesis. It is generally thought to be present in a constitutive form, COX-1, which participates in normal cellular functions, and an inducible form, COX-2 catalyzes the initial step of prostanoid synthesis by converting arachidonic acid (AA) into prostaglandin H_2. Prostaglandin H_2 functions as a substrate commonly used for many specific prostaglandin synthases. Induction of COX-2 has been shown to promote inflammatory responses by producing cytotoxic prostaglandins and ROS. Moreover, neuronal cytokines in the brain are known to induce COX-2. Several studies have also shown induction of COX-2 in RGC in response to elevated IOP [33]. In line with this, RGC can be blocked by COX-2 inhibition such as Dexibuprofen. Overall, COX-2 may cause neuronal damage through the induction of inflammatory responses [33]. In this sense, our group has designed Dexibuprofen drug delivery systems to be administered as eye-drops for ocular inflammation treatment [34]. These systems are being currently assessed in an *in vivo* glaucoma model (data not published).

Gonadal Steroids

Mital, et al. encapsulated gonadal steroids into PLGA NPs and they increased the bioavailability of the drug after oral administration up to 10 times compared with the free drug [35]. In this way, other authors encapsulate mifepristone in order to increase the bioavailability after oral administration [16].

The relationship between the endocrine secretion of the gonads and intraocular pressures is an established topic of speculation in the ophthalmic literature. To date, there are controversial reports about this issue. Obal and colleagues

claim an improvement in IOP values when using progesterone [36]. However, no recent publications claim gonadal steroids as a neuroprotection strategy for glaucoma.

α-, β-, and γ-secretase: Inhibitors Aβ

Aβ peptides originate from proteolysis of the APP by the sequential enzymatic actions of β-site APP-cleaving enzyme 1 (BACE-1, a β-secretase) and γ-secretase. Instead, the non-amyloidogenic pathway involves successive APP cleavages by α- secretase and γ-secretase, leading to the formation of non-amyloidogenic fragments [16].

Our research group is currently working on the encapsulation of epigallotechin-3-gallate, (EGCG) into polymeric NPs obtaining favorable results [37,38]. Other authors have demonstrating EGCG antioxidant and properties as well as its ability to promote non-amyloidogenic processing of APP by upregulating α-secretase preventing brain Aβ plaque formation. In addition, Smith and colleagues have also demonstrated EGCG entrapment into non-polymeric drug delivery systems targeted with tet-1 peptide effective for AD [29].

Cabaleiro-Lago, et al. reported the use of 40-nm-diameter poly (N-isopropylacrylamide)-co-poly(N-tert-butylacrylamide) (PNI- PAAM-co-PtBAM) NPs to hinder Aβ fibril formation in AD. The authors demonstrated that these NPs were able to interfere with the aggregation process by delaying the nucleation step, whereas no influence on the elongation step was noticed. More importantly, it was found that the oligomerization of the peptide could be reversed sufficiently where mature fibrils start forming. These co-polymeric NPs introduced a "lag phase" in between the nucleation and the elongation steps of the fibrillation. This "lag phase" was shown to be strongly dependent on the physicochemical characteristics of the NP surface and concentration [39].

PLGA NPs can also be functionalized with antibodies in order to increase the transport trough the BBB such as the study developed by Loureiro, et al. [40] where they encapsulate peptide iAβ5 into PLGA NPs surface functionalized with anti-transferrin receptor monoclonal antibody (OX26) and anti-Aβ (DE2B4) [40].

In addition, Yamamoto and colleagues have demonstrated *in vitro* that β-secretase could be a potential target for therapy of neurodegenerative retinal diseases [41].

Guo, et al. demonstrated that Aβ colocalizes with RGC in experimental glaucoma and induces significant RGC apoptosis *in vivo* in a dose- and time-dependent manner. In addition, they demonstrate that targeting different components of the Aβ formation and aggregation pathway can effectively reduce glaucomatous RGC apoptosis *in vivo* [42]. This opens a new window for possible treatments combining nanotechnological approaches in order to obtain a sustained drug delivery.

Antioxidant Species

Glutathione (GSH), a water-soluble endogenous antioxidant composed of glutamic acid, glycine, and cysteine is one of the most important intracellular antioxidants. In this sense, Reddy et al investigated the encapsulation of a metalloprotein, superoxide dismutase (SOD) into PLGA NPs increasing its circulating half-life, cell membrane permeability, and brain uptake. SOD is a free-radical scavenger that plays a key role in the major endogenous cellular defense mechanism against superoxide radicals. The authors described the efficacy of these nanomaterials to deliver SOD to human neuronal cells in vitro and to protect them from H_2O_2-induced oxidative stress [43].

Conclusions

Polymeric NPs could constitute a suitable strategy to treat AD and glaucoma. Several strategies using these colloidal systems for controlled drug release in order to overcome the BBB and the BRB while encapsulating neuroprotective drugs and deliver them into the target tissue. In this sense, there is a wide variety of drugs using different several preparation methods. These drug delivery systems open a window for future neuroprotective treatments being able to be transported through the BBB and BRB and at the same time avoiding possible drug adverse and toxic effects. It is expected that this type of drug delivery strategies will have a strong impact in terms of creating an innovative pharmacological product feasible to translate into human patients for the treatment of neurodegenerative diseases.

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Chapter 7: Pentacyclic Triterpenes in Table Olives: Determination of Their Composition and Bioavailability by LC-MS

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Abstract

The fruit of *Olea europaea* L. is particularly rich in pentacyclic triterpenes, being maslinic and oleanolic acid the most prevalent compounds with minor amounts of erythrodiol. These secondary plant metabolites have been described to exert beneficial effects on health, such as hepatoprotective, anti-diabetic, antiviral, cardioprotective and antitumor, among other activities. The present review summarizes our results on the content of pentacyclic triterpenes in table olives analyzed by LC-MS, and their bioavailability after the oral administration of this food to Sprague-Dawley rats.

Keywords: Maslinic acid; Oleanolic acid; Erythrodiol; *Olea europaea* L.; LC-MS; Bioavailability

Abbreviations: MVA: Mevalonic Acid; HPCL: High-Performance Liquid Chromatography; LC-MS: Liquid Chromatography Coupled To Mass Spectrometry; ESI: Electrospray Ionization; APCI: Atmospheric Pressure Chemical Ionization; MRM: Multiple Reaction Monitoring; TIC: Total Ion Chromatogram; MA: Maslinic Acid, OA: Oleanolic Acid; UA: Ursolic Acid.

Introduction

Triterpenes are one of the major classes of natural products that contain six isoprene units with the basic molecular formula of $C_{30}H_{48}$. According to the structures of their backbones, triterpenes are classified into acyclic, monocyclic, bicyclic, tricyclic, tetracyclic, and pentacyclic triterpenes [1]. Many of them occur in their free form, as well as glycosides (saponins) or other combinations [1]. with more than 20000 different compounds identified in nature to date [2]. The majority of these natural products are biosynthesized and accumulated in plants as secondary metabolites that contribute to their protection [3]. From ancient times, plants containing these secondary metabolites have been used in traditional medicine to treat different human diseases [4]. In the last decades, numerous studies have been carried out in order to elucidate the beneficial effects on health and their potential pharmacological use. Recently, numerous biological activities, such as antitumor, anti-inflammatory, anti-diabetic, antiviral and hepatoprotective, among others were attributed to these compounds [5-11].

Triterpenes

Triterpenes are formed in higher plants predomintantly, through the mevalonate pathway, named after its key intermediate, mevalonic acid (MVA) (Figure 1). The cellular endogenous substrate, acetyl-CoA, is transformed to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Then, HMG-CoA reductase, one of the most highly regulated enzymes in nature, catalyzes the synthesis of MVA, which is the precursor of the pathway [12,13]. The involvement of two kinases leads to the generation of isopentenyl diphosphate (IPP) that undergoes isomerization to dimethylallyl diphosphate (DMAPP) by the enzyme isopentenyl diphosphate isomerase. IPP and DMAPP are two key isoprene units given to their role as universal 5-carbon building blocks in the synthesis of terpenes [13].

For many years, IPP and DMAPP were thought to be exclusively formed via the MVA pathway. However, in the 1990s a species-specific second route was described and termed the 2-C-methyl-D-erythtritol-4-phosphate (MEP) pathway because MEP was formed from pyruvic acid and glyceraldehyde-3-phosphate as initial substrates [14]. Recent research demonstrated that the MVA pathway is solely involved in the synthesis of IPP and DMAPP in the cytosol of animals, fungi, archaea as well as a few bacteria, whereas the MEP route is exclusive of most other bacteria and parasitic apicomplexa [14].

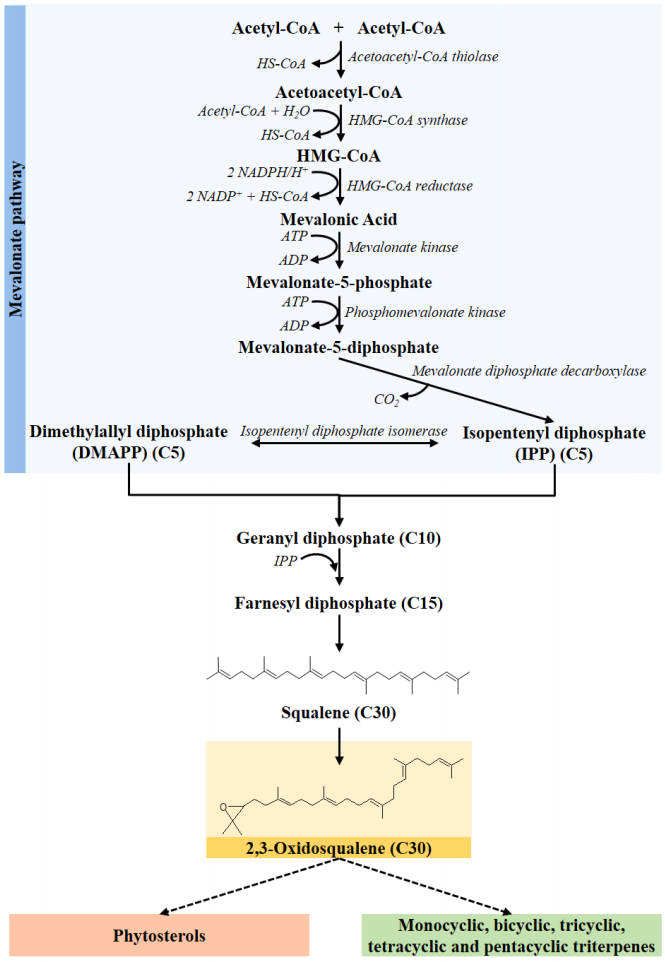

Figure 1: Biosynthesis of triterpenes through the mevalonate pathway in the cytosol of higher plants. Single arrows represent one step conversion, dashed arrows represent multiple steps.

In plants, both pathways have been described, but in independent compartments. The enzymes for the MVA pathway are located in the cytosol, whereas the ones for the MEP pathway are found in the plastids [13]. This pathway has been reported to synthesize the building blocks, mainly for the generation of monoterpenes (C10), diterpenes (C20), sesterpenes (C25), carotenoids (C40) and long-chain phytol [4]. Conversely, the cytosolic MVA pathway have been described to be responsible for the production of IPP and DMAPP mainly used in the formation of sesquiterpenes (C15), triterpenes (C30) and polyterpenes (>45) [4]. Although each route supplies IPP and DMAPP for the synthesis of the different terpenes, a metabolic crosstalk takes place between them [14].

The synthesis of triterpenes in plants takes place by a condensation reaction of 2 units of IPP and 1 unit of DMAPP to produce farnesyl diphosphate (C15) in a two-step process including the formation of the intermediate geranyl diphosphate Then, two units of farnesyl diphosphate merge to form squalene (C30) which serves as the precursor of acyclic triterpenes in plants [4].

Squalene undergoes an epoxidation reaction that enables the synthesis of 2,3-oxidosqualene (C30) which is the branch point between the pathway for the biosynthesis of primary

and secondary metabolites. The activity of oxidosqualene cyclases, also known as triterpenes synthases, catalyzes the ring forming reactions producing the diverse triterpenoids scaffolds, namely monocyclic, bicyclic, tricyclic, tetracyclic, and pentacyclic triterpenes [4]. Otherwise, the cyclization of 2,3-oxidosqualene to cycloartenol via cycloartenol synthase serves to the formation of membrane phytosterols and steroid hormones [4].

Pentacyclic Triterpenes

Pentacyclic triterpenes comprise different members, among which the oleanane, ursane and lupane groups are the most relevant due to their numerous biological activities, such as antitumor, anti-inflammatory, anti-diabetic, antiviral and hepatoprotective, among others [5-11]. Of them, the oleanane skeleton is the most abundant in higher plants, being oleanolic acid one of the most widely distributed triterpene in nature, and together with maslinic acid and in lesser amounts [1]. These compounds arises from ß-amyrin, leading to the formation, in first place, to the precursor erythrodiol, that is subsequently transformed to oleanolic and maslinic acids.

Erythrodiol or 3ß-olean-12-en-3,28-diol (Figure 2) has been found in very few species aside from *Olea europaea* L. where it has been detected in the fruit and oil in low amounts [3]. Despite the scarce information about this compound, erythrodiol has been reported to exert antitumor [15-17], anti-inflammatory [18] and cardioprotective activities [19,20].

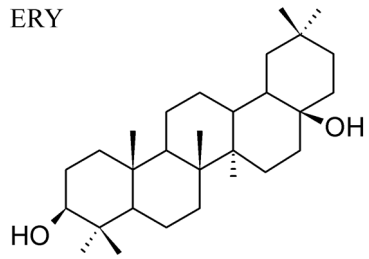

Figure 2: Chemical structure of erythrodiol (ERY).

Oleanolic acid or 3ß-hydroxy-olean-12-en-28-oic acid Figure 3. is formed after the oxidation of alcohol in the C28 position of erythrodiol. This compound has been isolated from more than 1600 species, including edible foods and medicinal plants [1,5]. Worth mentioning is the fact that this pentacyclic triterpene is prevalent in the Oleaceae family, especially in *Olea europaea* L., the plant species from which this compound has been named [21]. Oleanolic acid possesses prominent pharmacological activities, being the hepatoprotective, anti-inflammatory, antioxidant, anti-diabetic, and antitumor activities the most outstanding ones [22].

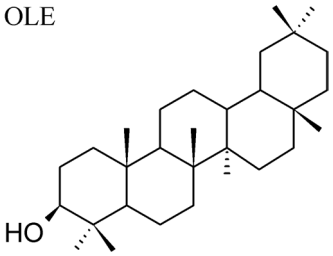

Figure 3: Chemical structure of oleanolic acid (OA).

Maslinic acid or 2α,3ß-hydroxy-olean-12-en-28-oic acid Figure 4. is synthesized from oleanolic acid differing only in an additional hydroxyl group at the 2-carbon position. Maslinic acid was isolated in 1927 from the leaves of Crataegus oxycantha L. and was named "crategolic acid" [23]. This compound has been detected in 30 species worldwide and it is one of the main pentacyclic triterpenes found in *Olea europaea* L. [24,25]. This compound has received less attention compared to the numerous studies devoted to its precursor oleanolic acid, despite exerting promising health-protecting properties, such as antitumor, anti-diabetic, antioxidant, cardioprotective, neuroprotective, antiparasitic and growth-stimulating [7].

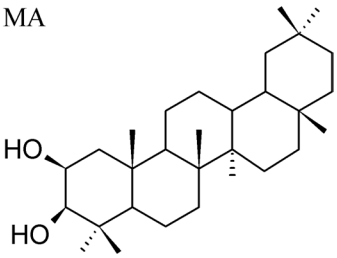

Figure 4: Chemical structure of maslinic acid (MA).

Natural Sources of Pentacyclic Triterpenes

Pentacyclic triterpenes from the oleanane type, namely maslinic and oleanolic acids, are ubiquitously distributed in higher plants Table 1.

Maslinic and oleanolic acids are located mainly in the intracuticular wax compartment of the cells [3]. The wax layer is one of the two main components of the cuticle that covers the surfaces of aerial parts, namely, leaves, flowers, fruits and non-woody stems, of all terrestrial plants forming the first protective barrier against abiotic and biotic environmental stresses [3]. Therefore, these compounds would be incorporated in the diet, principally with the ingestion of foods with edible peel [3] and have been described in numerous vegetables, legumes, species and fruits (Table 1). Maslinic and oleanolic acids have been

found in eggplants and spinaches [26], in legumes such as chickpeas and lentils [27] or in species such as basil [26] and rosemary [33]. These compounds are also present in fruits, such as grapes [3,30] or apples [28], the latter being one of the fruits most consumed worldwide and its antitumor effects have been correlated with its peel which contains both, oleanolic and maslinic acids [35].

Food	Maslinic acid	Oleanolic acid	References
Vegetables (mg/kg dry weight)			
Eggplant	840 ± 70	530 ± 40	[26]
Spinach	1260 ± 110	1670 ± 130	[26]
Carrot	n.d.	250 ± 40	[26]
Celery	n.d.	170 ± 20	[26]
Cooked legumes (mg/kg fresh weight)			
Chickpeas	61.9	3.56	[27]
Lentils	26.3-38.5	4.2-5.3	[27]
Pinto beans	n.d.	25.9	[27]
Fruits (mg/kg dry weight)			
Apple (fruit peel)	0.96 ± 0.03	3.18 ± 0.10	[28]
Apple pomace	10	139	[29]
Grapes (seeds)	10 ± 2	42 ± 3	[30]
Grapes (mg/kg fresh weight)	n.d.	30 – 160	[31]
Kiwi	17.3 ± 0.3	3.1 ± 0.1	[32]
Pomegranate	10.7 ± 0.4	n.d.	[32]
Lemon	3.4 ± 0.07	n.d.	[32]
Bilberry	n.d.	5.8-9.7	[3]
Aromatic herbs (mg/kg dry weight)			
Basil	350 ± 40	960 ± 70	[26]
Fennel	n.d.	540 ± 80	[26]
Rosemary	n.d.	31.6 ± 4.0	[33]
Brown mustard	330 ± 80	n.d.	[34]

Table 1: Content of maslinic and oleanolic acids in edible plants.

Consequently, in view of their wide distribution in edible plants (Table 1) the consumption of a diet rich in vegetables and fruits, such as the dietary pattern followed along the Mediterranean basin that has been associated with a lower incidence of cancer along with other chronic diseases [36] could provide a constant supply of these phytochemicals, besides other nutraceuticals with health protecting activities.

Plants used in traditional medicine to treat diverse ailments contain also these pentacyclic triterpenic acids. *Lagerstroemia speciose* or banaba has been widely employed as tea and herbal remedy since ancient times for the treatment of diabetes [37]. The leaves are specially rich in maslinic acid with concentrations of 4.96 ± 0.13 mg/g followed by oleanolic acid with 0.82 ± 0.03 mg/g [30]. Maslinic and oleanolic acids isolated from this specie acted as α-glucosidase inhibitors with IC_{50} of 5.52 ± 0.19 and 6.29 ± 0.37 μg/mL, respectively. *Syzygium aromaticum* or clove, also used for its hypoglycemic activities, contains both compounds that were demonstrated to down-regulate the increase of SGLT1 and GLUT2 expressions in the small intestine of STZ-induced diabetic rats, and also inhibited small intestine α-amylase, sucrase and α-glucosidase activity [38]. *Crataegus monogyna* L., commonly known as hawthorn, contain 0.93 ± 0.01 mg/g and 2.34 ± 0.11 mg/g of maslinic and oleanolic acids, respectively [30]. This plant exerts hypotensive, antioxidant, anti-inflammatory, and vasodilating effects, and has been traditionally used to strengthen cardiovascular function.

Ortosiphon stamineus L. possesses several pharmacological activities such as diuretic, hepatoprotective,

anti-diabetic, antihyperlipidemic, and has been described to contain 0.84 ± 0.06 mg/g and 2.77 ± 0.19 mg/g of maslinic and oleanolic acids, respectively [30]. Moreover, these pentacyclic triterpenes have been reported in *Eriobotrya japonica*, employed as antitussive and anti-inflammatory for chronic bronquitis [39], in *Geum japonicum* utilized as diuretic [40], and *Agastache rugose* applied in the treatment of intestinal disorders [41].

Synthesis of Pentacyclic Triterpenes in *Olea europaea* L.

Olea europaea L. is a prevalent species in human nourishment that has been cultivated for more than 5000 years in the countries bordering the Mediterranean Sea. The olive tree has been highly appreciated since ancient times [42]. This plant is characterized for being resistant and productive thus providing a versatile and valuable crop. For centuries, table olives and olive oil have been a pivotal component of the diet consumed along the Mediterranean shore [42,43]. Consequently, *Olea europaea* L. has traditionally provided important economic and dietetic benefits for the people of the region. Moreover, olive oil and leaves have been widely used in traditional medicine, standing out the use of the olive leaves as hypoglycemic, antihypertensive, antimicrobial, and antiatherosclerotic among others [43]. The by-products of *Olea europaea* L. have long been known to contain a wide range of bioactive compounds, including high amounts of pentacyclic triterpenes that were first identified in olive pomace [44], and later in the fruit [45]. The synthesis of this group of compouds has been comprehensively studied in *Olea europaea* L. by Stiti, et al. [46] which has postulated a biosynthetic pathway from 2,3-oxidosqualene.

In the olive tree, the production of pentacyclic triterpenes requires the generation of different carbocationic intermediates. Therefore, the cyclization of 2,3-oxidosqualene leads to the formation of the tetracyclic dammarenyl cation that is further transformed to the pentacyclics baccherenyl and lupenyl intermediates prior to the formation of the oleanyl and ursanyl cations (Figure 5) [46]. The final products are due to the stabilization of specific carbocationic intermediates which are further metabolized into more-oxygenated compounds mainly through reactions catalyzed by cytochrome (CYP) 450 enzymes [47].

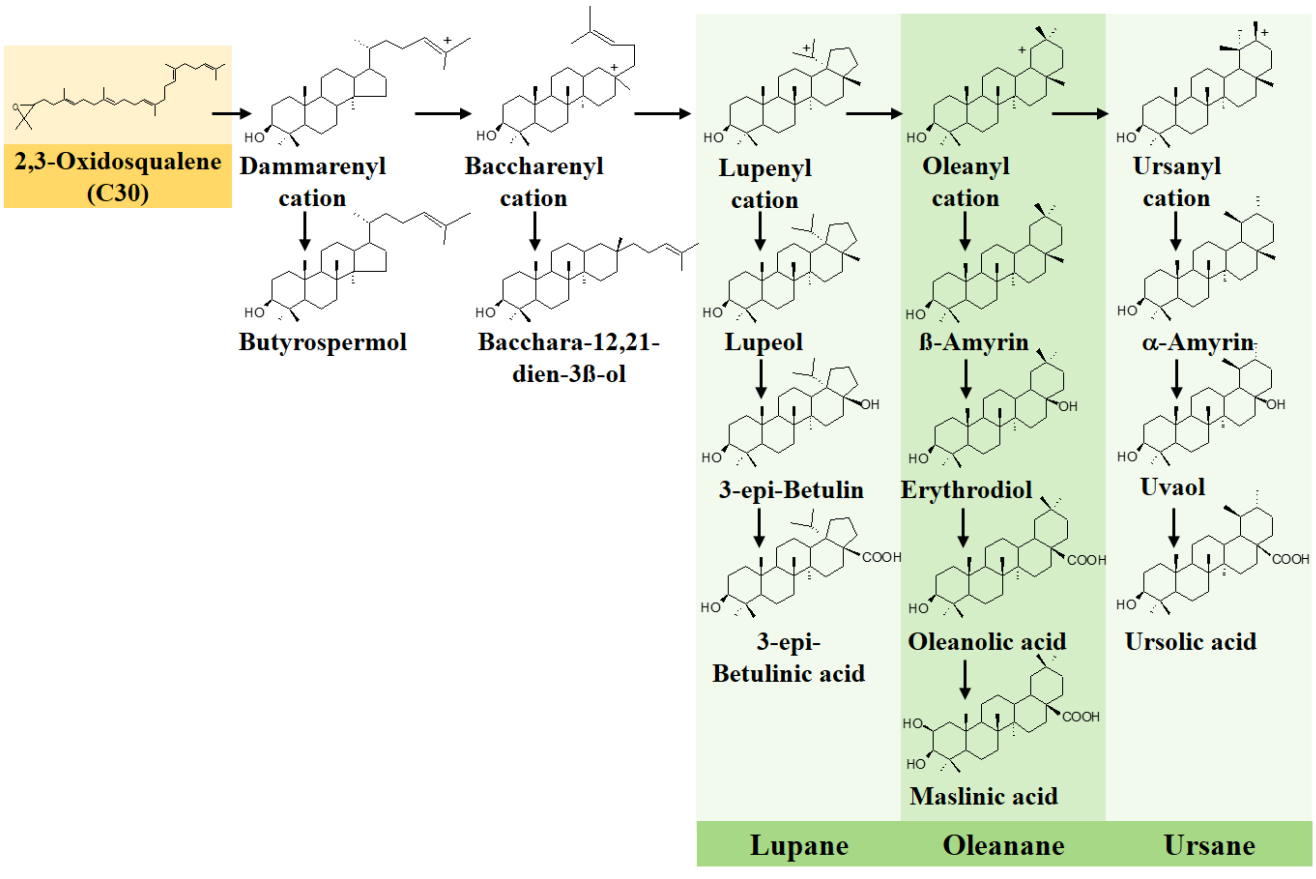

Figure 5: Synthesis of pentacyclic triterpenes in *Olea europaea* L.

In *Olea europaea* L., the main pentacyclic triterpenes arises from the stabilization of the oleanyl cation to ß-amyrin (olean-12-en-3ß-ol). This compound is sequentially oxidized at the C_{28} position by a cytochrome (CYP) P450 enzyme to yield in first place the alcohol erythrodiol and in second place oleanolic acid [4]. Finally, a different CYP P450 catalyzes the addition of a hydroxyl group forming maslinic acid [47]. The pentacyclic triterpenes from the oleanane group are produced in higher amounts than those of the ursane or the lupane classes [46]. Stiti, et al. [46] analyzed the pentacyclic triterpenes in the olive, and evaluated their content throughout fruit ontogeny (Table 2). Although these authors identified 19 pentacyclic triterpenes arising from different carbon skeletons, they demonstrated that oleanane triterpenoids were largerly predominant, representing the 99.2% of the different triterpenoids in olives [46].

Pentacyclic triterpenes	mg/Kg	%	Reference
Oleanane group			
ß-Amyrin	4	0.16	[46]
Erythrodiol	13	0.52	[46]
Oleanolic acid	946	38.08	[46]
Maslinic acid	1502	60.46	[46]
Ursane group			
α-Amyrin	n.d.	--	[46]
Uvaol	0.4	0.02	[46]
Ursolic acid	4	0.16	[46]
Lupane group			
epi-Betulin	0.8	0.03	[46]
epi-Betulinic acid	14	0.56	[46]

Table 2: Pentacyclic triterpenes in olives of the Chemlali variety harvested 30 weeks after flowering and analyzed by gas chromatography - mass spectrometry.

Distribution of Pentacyclic Triterpenes in *Olea europaea* L.

Pentacyclic triterpenes are synthesed in *Olea europaea* L. as secondary metabolites required for the plant survival in its environment [3]. These compounds have been described in the epicuticular waxes of olive fruits [45] and leaves [48] and their protective role has been associated with the development of a physical barrier that prevents of water loss and acts as first defense against pathogens [3].

Food	Maslinic acid	Oleanolic acid	References
Table olives (mg/kg fresh weight)			
Manzanilla, plain black	287.1 ± 66.6	178.8 ± 43.7	[49]
Manzanilla, plain green	384.1 ± 50.0	202.6 ± 57.3	[49]
Hojiblanca, plain green	904.7 ± 259.6	565.2 ± 107.1	[49]
Gordal, plain green	414.2 ± 89.3	294.3 ± 4.5	[49]
Kalamata, plain natural black	1318.4 ± 401.0	841.4 ± 162.9	[49]
Conservolea	1349 ± 123	536 ± 82	[50]
Olive oil (mg/kg)			
Olive oil, extra virgin	19-98	17-85	[51]
Olive oil, virgin	145-251	167-356	[51]
Pomace oil	575-698	405-703	[52]

Table 3: Content of maslinic and oleanolic acids in *Olea europaea* L.

In the fruits of *Olea europaea* L. the formation of the oleanane type of pentacyclic triterpenes is predominant with respect to the ursane group. Up to now, the studies in the literature had only measured pentacyclic triterpenic acids in table olives, being maslinic acid found in higher concentrations than oleanolic acid. However, the content depends on different factors, such as the variety, cultivar, climate, degree of ripening on the time of harvesting but also on the method of elaboration of olives and post-fermentation conditions.

Virgin olive oil is obtained in a process involving pressing, which may disrupt the surface waxes on the fruit. Therefore, part of maslinic acid contained in the olive may be transferred to the oil. However, the amount of maslinic and oleanolic acids in the oil is much lower than in the fruit and depends on the oil quality (Table 3).

Lately, olive leaves have raised much attention due to the high presence of different families of bioactive compounds that could be used as raw material for the obtainment of high added value compounds of use as functional foods, drugs or cosmetics. Therefore, recent research have reported that the leaves contain pentacyclic triterpenes arising from both ß-amyrin and α-amyrin [24,48,53], being especially rich in oleanolic acid that accounts for a 54-76% of all the triterpenes depending on the cultivar [24,48,53]. The concentrations in Arbequina, Hojiblanca and Picual have been reported to range from 29.2 mg/kg in Arbequina [24] to 39.8 mg/kg in the Picual variety [53]. Maslinic acid is the second compound in terms of concentration, followed by ursolic acid and the dialcohols, erythrodiol and uvaol (Table 4).

Pentacyclic triterpenes	g/kg dry weight	References
Oleanane group		
Erythrodiol	1.86-4.39	[24,48,53]
Oleanolic acid	13.0-39.8	[24,48,53]
Maslinic acid	1.91-7.30	[24,48,53]
Ursane group		
Uvaol	1.81-5.15	[24,48,53]
Ursolic acid	1.99-4.90	[24,48,53]

Table 4: Pentacyclic triterpenes in olive leaves from different cultivars.

Analysis of Pentacyclic Triterpenes

Pentacyclic triterpenes from the oleanane group have been reported to possess important beneficial effects on health [5-11] and their content in table olives cannot be disregarded. This food is regularly consumed not only in the countries where the cultivation of *Olea europaea* L. has been performed from ancient times, but also worldwide, due to the increasing interest in healthy eating to improve health and quality of life. However, one of the drawbacks in recommending the intake of a precise number of olives is the lack of knowledge on their content of pentacyclic triterpenes.

Pentacyclic triterpenes have been traditionally analyzed by gas chromatography although their high molecular weight along with low volatility require a derivatization step prior to its determination in table olives [24], olive oil [51] and commercial botanicals and food supplements [30]. To avoid the preliminary derivatization of analytes and a lower laboriousness of sample preparation and analysis, more recently, high-performance liquid chromatography (HPLC) was introduced in the determination of these compounds from olives [49,54]. However, HPLC coupled to UV or diode-array detectors holds the disadvantage of low UV absorption provided by their saturated skeleton, which leads to high limits of quantification. Sensitivity was improved by derivatization as performed for the analysis of pentacyclic triterpenic acids in fruits [26] or the analysis of food samples [55]. In addition, HPLC leads to long chromatographic runs, broadening chromatographic peaks and, as a result it generates an additional loss of sensitivity. The use of gradient elution does not completely resolve the problem, given that on the one hand, it could reduce retention times, but on the other, a loss of resolution for structurally close isomers is observed. Therefore, the methods used to analyze pentacyclic triterpenes hold several weaknesses that difficult the accurate analysis of pentacyclic triterpenes in olives, none of them allow the simultaneous analysis of the acids (maslinic and oleanolic acids) with the alcohol (erythrodiol), as well as the concurrent determination of the pentacyclic triterpenes from both, the ß-amyrin and α-amyrin classes.

Determination of Pentacyclic Triterpenes in Table Olives by LC-MS

The determination of pentacyciclic triterpenes in table olives was a challenging task, not only for being contained in a complex matrix, but also, for the possible presence in the

samples of two pairs of positional isomers, namely, oleanolic acid and ursolic acid as well as erythrodiol and uvaol.

Although in *Olea europaea* L. the predominant pathway in the formation of pentacyclic triterpenes is via ß-amyrin formation, the fact that derivatives from α-amyrin could be present cannot be underestimated. Hence, the most important problem in existing approaches to the determination of pentacyclic triterpenes is the separation of analytes. To overcome these shortcomings, liquid chromatography coupled to mass spectrometry detection (LC–MS) has become a powerful hyphenated technique that enables the separation, unambiguous detection and characterization of bioactive compounds in complex samples. Therefore, we developed a selective and sensitive LC–MS method for the simultaneous determination of maslinic, oleanolic and ursolic acids, as well as erythrodiol and uvaol, the main triterpenic compounds present in *Olea europaea* L. [25].

The separation of the isomers was attempted instead of using a traditional octadecyl silica column, by the use of the stationary phase designed for the analysis of polycyclic aromatic hydrocarbons with polymeric C18 bonding. This Zorbax Eclipse PAH (Agilent Technologies) column was used due to its well-known resolution power towards geometric isomers. The combination of these novelty stationary phase along with the use of an isocratic mobile phase consisting of methanol 83% and water 17% allowed the adequate separation of oleanolic acid from ursolic acid, and erythrodiol from uvaol. Moreover, the simplicity of the mobile phase, that did not use any modifier avoided the formation of adducts when coupled to mass spectrometry.

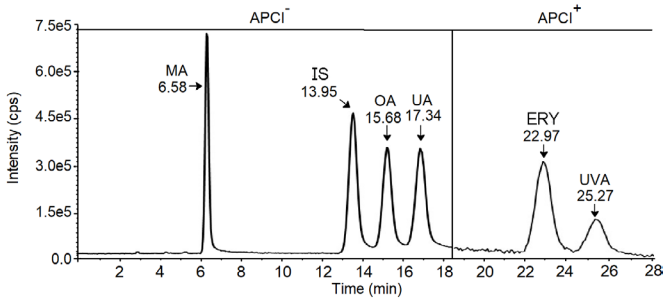

Figure 6: Representative total ion chromatogram (TIC) obtained by LC-APCI-MS of maslinic acid (MA), oleanolic acid (OA), ursolic acid (UA), erythrodiol (ERY) and uvaol (UVA) dissolved in methanol 80% at 2.5 µM and betulinic acid (IS) at 2 µM.

Once the chromatographic separation was established, we optimized the conditions for the detection of these compounds by MS. Several authors had proposed electrospray ionization (ESI) for the analysis of pentacyclic triterpenes [48,56,57], however although triterpenic acids were ionized in negative mode, this ionization source proved to be completely inadequate for the alcohols, erythrodiol and uvaol, that failed to be detected neither in positive or in negative polarity. Hence, atmospheric pressure chemical ionization (APCI) that has been described to provide a more effective ionization of low polarity compounds was evaluated. APCI in negative mode was selected for the analysis of triterpenic acids, since it gave a sensitivity two order of magnitude higher than negative ESI or positive APCI (Figure 6). On the other hand, erythrodiol and uvaol were only detected with the APCI source set in positive mode Figure. 6.

Fragmentation of pentacyclic triterpenes was evaluated in the LC–QqQ-MS to perform multiple reactions monitoring (MRM) analysis. The obtained MS/MS transitions were as follows: maslinic acid 471.3 → 393.3 and 471.3 → 377.3; oleanolic and ursolic acids 455.3 → 407.3, as well as erythrodiol and uvaol, 425.3 → 191.3. These transitions are consistent with the ones indicated by Paragón [54] in table olives and Sánchez-Avila, et al. [48] in olive leaves. Nevertheless, pentacyclic triterpenes were poorly fragmented even at high values of collision energy, resulting in peaks of low intensity. Then, the limits of quantification (LOQ) in MRM mode ranged from 125 to 650 nM for the five analytes and were two orders of magnitude higher than those achieved in single ion monitoring mode (SIM). The low sensitivity observed in MRM detection was in agreement with the valued obtained for Sánchez-Ávila, et al. [48] that gave values ranging from 190 to 650 nM. Although MRM mode allows a reliable identification of analytes, the fact that the isomers hold the same molecular weight, along with the same MS/MS transitions does not improve the selectivity already accomplished in the chromatographic separation. Therefore, pentacyclic triterpenes were detected using SIM mode in order to achieve the highest sensitivity in the analysis [25].

Once the LC-MS conditions were established, the developed method was validated using calibration standards following the EMA Guidelines on Bioanalytical Method Validation [58]. Excellent sensitivity was achieved with limits of detection for the triterpenic acids lower than 1 nM, whereas for erythrodiol and uvaol were 4.5 and 7.5 nM, respectively. The method was linear for the five analytes in the range of concentrations from 0.005 to 15 µM with correlation coefficients exceeding 0.99. The precision and accuracy were ≤ 9.90% and ≤ 9.57%, respectively [25].

The validated method was applied to the determination of pentacyclic triterpenes in table olives, since the fruit of *Olea europaea* L. constitutes an example of food rich in those bioactive molecules. However, prior to the analysis, the extraction process was optimized in terms of type of solvent, by spiking olive samples with MA and OA and evaluated their

recovery. The best recovery was achieved when a mixture of ethanol:methanol (50:50%) was employed

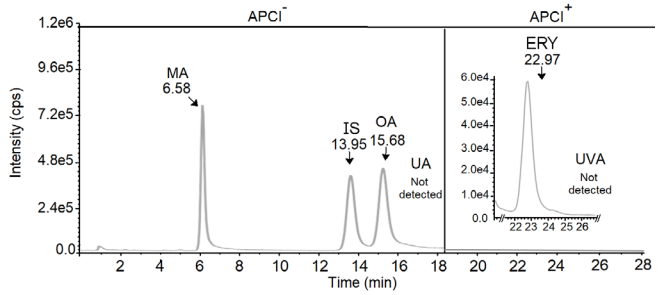

Figure 7: Representative total ion chromatogram (TIC) obtained by LC-APCI-MS of the pentacyclic triterpenes detected in table olives from the Marfil variety. MA, maslinic acid; OA, oleanolic acid; UA, ursolic acid; ERY, erythrodiol; UVA, uvaol; IS, internal standard. Erythrodiol was detected in non-diluted samples and is depicted in an insert.

Overall, the extraction process established for the analysis of pentacyclic triterpenes in table olives was quite straightforward, holding minimal sample pre-treatment. In first place, olives were pitted, and the destoned fruit was grinded in order to break down the tissue and yield a fine and consistent suspension. This homogenization facilitated the second step, which consisted on three cycles of extraction, with the first one with 12 mL of ethanol:methanol and the repetitions only involving 6 mL of solvent. Finally, the pooled supernatants were diluted and directly analyzed by LC-MS [25].

The developed method was applied to the analysis of Marfil table olives which are a scarcely studied variety distinguished by an ivory hue from which it takes its names and native to the Montsià region (Tarragona, Spain). These olives were processed following the Greek-style, which consist in a natural fermentation in brine. The developed method enabled the identification and quantification analytes derived only from the ß-amyrin, namely, maslinic and oleanolic acids, as the main pentacyclic triterpenes and erythrodiol in minor amounds (Figure 7). Ursolic acid and uvaol were not found Marfil table olives.

The content of maslinic and oleanolic acids found in the Marfil variety, processed as natural green olives (Table 5), is consistent with those described by Peragón [54] and Romero, et al. [49] also for fruits that followed a natural fermentation process, such as Kalamata and Hojiblanca. These results agree with other authors which reported the presence of the derivatives from α-amyrin in olive leaves but not in the fruit [24,54].

Pentacyclic Triterpenes	mg/kg	%
Oleanane group		
Maslinic acid	1740 ± 60	55.4
Oleanolic acid	1380 ± 100	44
Erythrodiol	18.0 ± 1	0.6
Ursane group		
Ursolic acid	n.d.[1]	0
Uvaol	n.d.	0

Table 5: Pentacyclic triterpenes in Marfil table olives.
[1] n.d. Not detected

Determination of Pentacyclic Triterpenes in Plasma by LC-MS

Based on our results, the scarcely known Marfil variety of table olives emerges as an important source of bioactive compounds [25]. However, there was a lack of knowledge on the absorption, metabolism and distribution either in humans or in animals of pentacyclic triterpenes after the consumption of table olives. To carry out these bioavailability studies, the first step consists in the development of an analytical method able to detect maslinic acid, oleanolic acid and erythrodiol that could reach the blood after the oral intake of olives. For the LC-MS determination of pentacyclic triterpenes, the conditions previously developed for the analysis of table olives were used since they provided enough sensitivity for the accurate detection of the compounds in plasma [25]. However, a different extraction process should be applied, given that plasma and table olives are quite different matrixes. Consequently, the extraction of pentacyclic triterpenes from plasma was attempted using a previously developed method for the analysis of maslinic acid [59]. The method consisted on two consecutive extractions with ethyl acetate followed by evaporation to dryness and reconstitution with methanol 80% prior to LC-

MS analysis. Previous to the implementation of the method, it was validated in order to evaluate if the conditions were adequate for the extraction of oleanolic and ursolic acids as well as erythrodiol and uvaol. Consequently, blank rat plasma samples were spiked using three concentration levels in order to validate the analytical performance following the EMA Guidelines [58]. Linearity was confirmed with calibration curves that gave correlation coefficients above 0.99. Regarding accuracy and precision, evaluated as intra-day and inter-day reproducibility, the method showed adequate RSD (%) values (0.22–9.93%) lower than others [60-62]. In addition, no significant matrix effect for analytes and IS were observed as values were around 91–117%, being within the 80-120% range that indicates that the ionization competition between the analyte and endogenous co-elutions was negligible and the LC–MS method was robust. Recoveries were around 100% for all the evaluated analytes. Moreover, the LOQ was adequate since it ranged from 1 nM for maslinic acid to 10 nM for uvaol, thus providing similar [60-63] or higher sensitivity [56,64] in comparison with the published methods. Therefore, the proposed methodology represents an important achievement and opens the possibility to bioavailability studies after consumption of different foods, or administration of plants widely used in traditional medicine, with the aim of studying in depth the beneficial effects of these compounds in human beings [65].

The analytical performance of the method was verified by administering a finely grinded suspension of Marfil olives to Sprague-Dawly rats. The dose to be administered to the experimental animals was established bearing in mind that it should be kept within a nutritional range, and olives should be finely grinded so that they could be administered intragastrically with a cannula. For this reason, approximately 12 g of destoned fruit was grinded with 40 mL of water, and the experimental animals were administered with this finely minced suspension at the volume of administration of 10 mL/kg. The dose that the rats received corresponded to 3 g of destoned olives/kg body weight. This dose was translated to the equivalent to be ingested by a person with the body surface area normalization method described by Reagan-Shaw, et al. [66]. The equivalent dose for a human corresponded to 0.48 g/kg, which means that a person of 70 kg of body weight would consume 28 olives of the Marfil variety to accomplish the dose administered to rats. Although this dose does not correspond to the amount of olives eaten in a meal or as appetizer, it is not so different from the one usually consumed and would be compatible with the dose to be administered in future pharmacokinetic studies using olives.

Plasma samples obtained 120 min after the oral administration of olives from the Marfil variety indicated the presence of maslinic acid at 23.1 ± 5.3 nM and oleanolic acid at 4.32 ± 0.20 without traces of erythrodiol. The fact that the latter could not be found in plasma, can be attributed to its low content in the fruits of *Olea europaea* L. that was two orders of magnitude lower than maslinic acid and oleanolic acid. The relatively low concentration obtained for maslinic acid (~ 25 nM) after the administration of the suspension of olives that contained an approximate dose of 4.57 mg maslinic acid/kg of rat body weight, could be explained by the described oral bioavailability of approximately 5% obtained in rats [67]. On the other hand, oleanolic acid was administered in the form of a suspension at a dose of 3 g of destoned olive/kg which contained 3.60 mg of oleanolic acid/kg of body weight was detected at concentrations around 5 nM. Previous studies in the literature that administered oleanolic acid to rats, either as a single compound or in part of an extract, indicates the poor oral bioavailability of this pentacyclic triterpene [63,68,69] that has been described to be 0.7% for oral doses of 25 and 50 mg/kg [68]. Therefore, the lowest bioavailability described for oleanolic acid could explain our results, in which this pentacyclic triterpene was barely detected in comparison to maslinic acid. Hence, the oral administration of olives to rats and its determination in plasma verified that the established methodology is appropriate for bioavailability studies.

Conclusion

Pentacyclic triterpenes from the oleanane group, mainly maslinic and oleanolic acids, have received much attention in the recent years due to their numerous biological activities, such as antitumor, anti-inflammatory, anti-diabetic, antiviral and hepatoprotective, among others. These compounds are widely distributed in nature, in both medicinal species and edible plants, especially in vegetables, legumes and fruits regularly consumed following a Mediterranean dietary pattern. Among the foods rich in pentacyclic triterpenes from the oleanane group stands out the fruit of *Olea europaea* L. The present chapter presents the optimization and validation of two analytical strategies that allows the determination of these bioactive compounds in table olives and in plasma samples after the administration of this food. Remarkably, both extraction processes allowed a fast sample treatment prior to LC–MS analysis that allows a sensitive and reliable detection of pentacyclic triterpenes from the oleanane and ursane family as indicated in the validation of the method. The results obtained in table olives confirms this food as a prominent source of maslinic and oleanolic acids. Moreover, the administration of this food to rats and its subsequent plasmatic analysis allows the confirmation of the bioaccessibility of these compounds from table olives. Furthermore, the application of the analytical methods could be extended to other foods or plants used in traditional medicine, thus broadening the knowledge of these bioactive triterpenoids.

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